STUDIES ON THE NUTRITION OF DROSOPHILA WITH PARTICULAR REFERENCE TO NUCLEIC ACID METABOLISM

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Thesis submitted to the Faculty of Science, University of Edinburgh, in fulfillment of the requirements for the degree, Doctor of Philosophy

1955

Actually submitted 1956

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INTRODUCTION

A major portion of the progress in biological investigation within modern times has been directed at a clearer concept of life in terms of its underlying biochemical and biophysical processes. The study of metabolism within living organisms has been prompted by the theoretical consideration that the end result - animal, plant or virus - must be in part the result of numerous, often interacting, biochemical reactions functioning within the cells and tissues of these organisms or within their hosts. In the minds of many investigators this consideration has been coupled with another: that heredity, from a physiological standpoint, is the transmission from one generation to the next of like forms of metabolism. To this end, biochemical investigations have been made on a variety of organisms, and many chemical and physical techniques have been brought to bear on the general problem of the inheritance of metabolic patterns underlying the structural details of the organism.

Some Aspects of Biochemical Genetics

There are a number of competent and extensive recent reviews and discussions in the field that has become known as biochemical genetics (e.g., Beadle 1945, 1946 and 1951; Haldane 1954; Wagner and Mitchell 1955), and it would be a

presumption on the part of the present author to undertake a similar survey of this entire area of research. The investigative efforts encompassed within the works cited above represent noteworthy attempts to correlate biochemical analyses with genetic data. Our ignorance in one field or the other has made success in this area most variable and largely dependent upon the suitability of the material used for both biochemical and genetic exploitation. At one extreme are instances where the biochemical aspects of the problem are explorable, but where direct proof of inheritance is impossible. The following are examples of this type of problem. 1. Nutritional deficiencies in the fungi, Penicillium notatum and P. chrysogenum, are known but these organisms have no sexual stage of development, prohibiting genetic analysis (Bonner 1946a). 2. The complete nutritional requirements of the ciliate Tetrahymena pyriformis (geleii) W have been elucidated, but this animal does not undergo conjugation or show any signs of sexuality (Kidder and Dewey 1951; see, however, Elliott and Gruchy 1952; Elliott and Hayes 1953; Nanney 1953; Nanney and Caughey 1953; and Nanney and Caughey 1955 for evidence of conjugation in other strains of Tetrahymena pyriformis). At the opposite end of the spectrum are a number of examples of complete understanding of the inheritance of characteristics having strongly assumed, but at present largely unknown, biochemical bases. Examples of this type are:

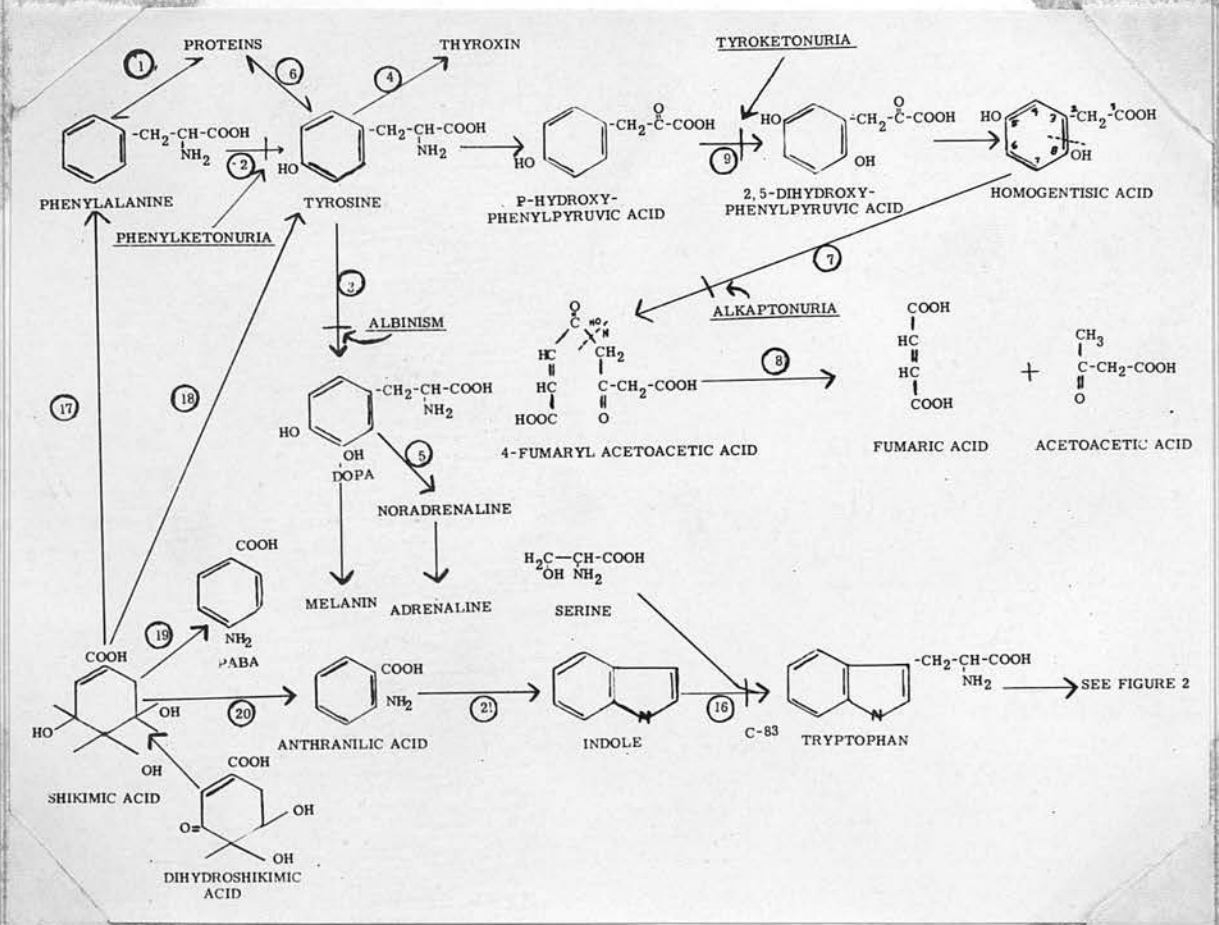
1. mammalian coat color inheritance (Wright 1941 and 1942); and 2. the inheritance of immunological specificity (Irwin 1951 and others). Between these two extremes lies a variety of examples representing all degrees of success and difficulty such as: 1. metabolic blocks in man; 2. insect eye color pigments; 3. flower color pigments; 4. sex phenomena in unicellular organisms; 5. amaylase inheritance in the silkworm, and many others. All of these converge on the work performed with the bread mold, Neurospora, which perhaps represents the ideal material from both biochemical and genetic standpoints.

One aspect of the search for understanding in the field of biochemical genetics has been a study of what the British biochemist and physician, A. E. Garrod, called "inborn errors of metabolism". These are unit processes within a complex system of metabolism which are inherited from generation to generation and, like the visible mutations of classical genetics, involve observable deviations from the normal pattern of events which can be measured in some way. All organisms thus far studied, including the viruses, require twenty or more amino acids for their maintenance, growth and development. Some organisms (autotrophic) are capable of synthesizing these amino acids from inorganic starting material, whereas others (heterotrophic) require some of them already preformed in their diet. Given these compounds, a

heterotrophic organism is capable of converting them to other amino acids through a series of metabolic reactions. Figures 1 and 2 are compilations of such reactions involving the aromatic amino acids known, or postulated, to occur in several organisms. The entire pattern is not known to occur in any single animal or plant, although some of the reactions are held in common by different species. Details of this pattern will be considered later.

It is known that man requires phenylalanine in his diet (Rose, Haines, Johnson and Warner 1943). Given phenylalanine, he is able to: 1. build it into his own protein (see reaction 1, Fig. 1) or 2. oxidize an excess to form tyrosine (reaction 2, Fig. 1). Tyrosine itself is derived from two sources: 1. the diet and 2. the conversion of phenylalanine. This latter source is known to provide for the majority of the metabolic requirement for tyrosine (Moss and Schoenheimer 1940). These authors were able to show that the conversion of phenylalanine to tyrosine occurred even when an excess of tyrosine was fed to rats. It would seem that the conversion was obligatory for the catabolism of phenylalanine. Thus, phenylalanine is known as an "indispensable" or "essential" amino acid whereas tyrosine is "dispensable" or "non-essential"; given an adequate supply of phenylalanine the organism can satisfy its need for tyrosine from it (Moss and Schoenheimer, op. cit.). Apparently the reaction is irre-

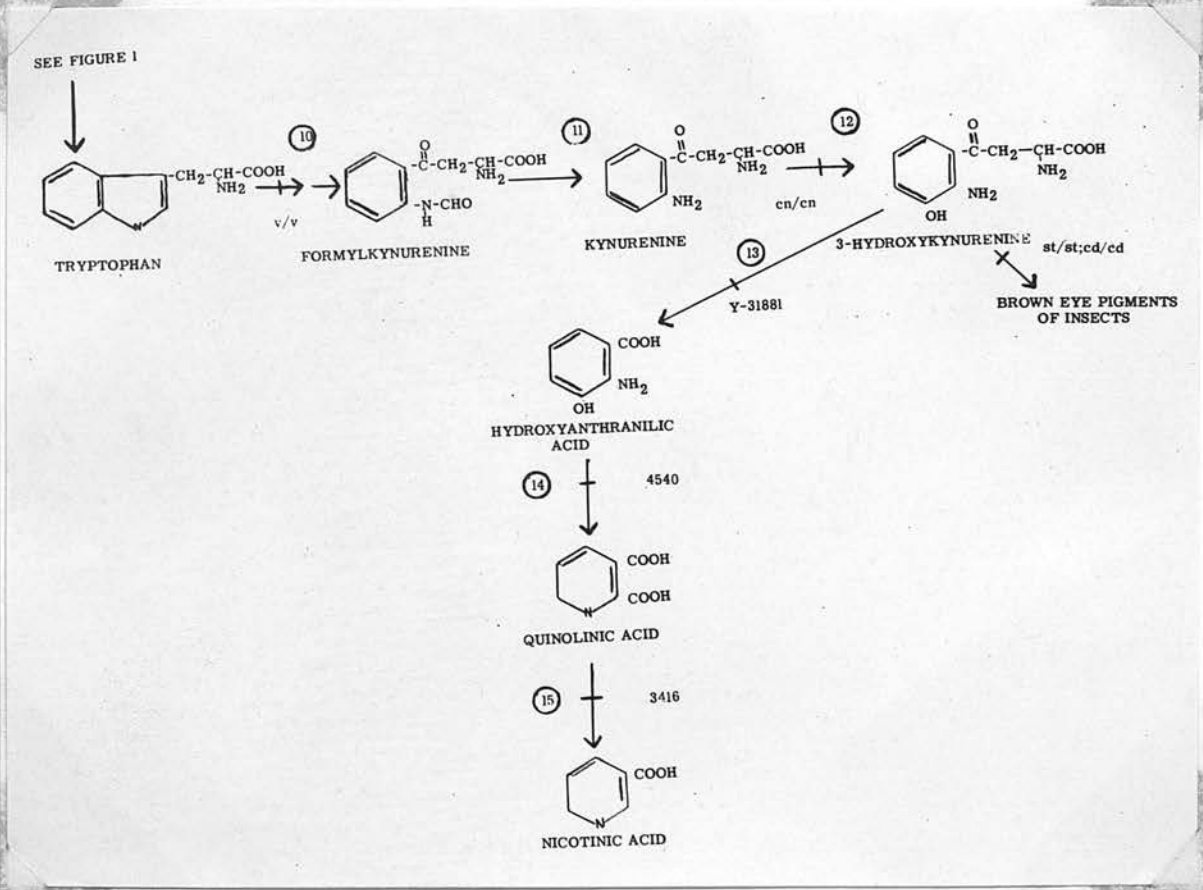
Figure 1



Aromatic Amino Acid Metabolism in Several Different Organisms

(see text for explanation)

Figure 2



Aromatic Amino Acid Metabolism in Several Different Organisms

(see text for explanation)

versible in that tyrosine fails to fulfil the requirement for phenylalanine in the growth of rats (Womack and Rose 1934) and fails to increase the blood level of phenylalanine in dogs fed the compound (Hier 1947).

Tyrosine stands as a pivotal compound in a whole array of reactions in man and other animals. It serves as a precursor for melanin formation (reaction 3, Fig. 1), for thyroxin formation (reaction 4, Fig. 1) and adrenaline and noradrenaline formation (reaction 5, Fig. 1). It also can be used in the building up of the protein of the body (reaction 6, Fig. 1), or it can be eventually broken down to carbon dioxide and water. It is obvious that to call an amino acid "dispensable" or "non-essential" simply refers to its mode of origin in the body and does not refer to its dispensability to the well-being of the organism.

These reactions into which phenylalanine and tyrosine enter, while not perfectly understood in man, have been studied from both a biochemical and a genetic standpoint in man as well as other organisms. They have been chosen, for the purposes of this introduction, as illustrative material for the explanation of fundamental concepts and ideas employed in the field of biochemical genetics.

It was Garrod (1902 et seq.) who first described in an extensive manner certain defects of phenylalanine-tyrosine metabolism and their mode of inheritance in man. In view of

the wealth of experimental data gained since that time, from the utilization of microorganisms in particular, it seems surprising that these first examples in the field were from a study involving human beings. The most famous, perhaps, of these metabolic defects is known as "alkaptonuria", originally described by Scribonius in 1584 (not available to the author, cited by Garrod 1923). Usually, this defect causes little hindrance to individuals who carry it, although it may be associated in later life with a disease known as "ochronosis" in which the cartilages, the sclerotic coat of the eyes, and the tendons become blackened with pigment. The most characteristic feature of alkaptonuria, however, is a darkening of the urine upon exposure to air. The substance present in the urine responsible for this darkening is 2,5-dihydroxyphenylacetic acid. This substance was originally isolated in crystalline form from alkaptonuric urine by Marshall (1887, cited by Garrod 1923) and named by him "glycosuric acid". Later, Wolkow and Baumann (1891) gave the substance its empirical formula and called the compound "homogentisic acid". The name "alkapton", formulated from the Arabic root algali and Greek root hapto by Bödeker in 1859 (cited by Garrod 1923) to describe its reducing property in certain alkaline copper solutions, has been widely used for this same compound.

Darwin (1868) was aware of the possibilities of parental inbreeding as cause for the occurrence of certain congenital

defects in humans, but Garrod (1902) was the first to study the parentage of individuals possessing such traits. He found a much higher proportion of consanguinity in the families exhibiting alkaptonuria than in the population at large. In England, it has been estimated that marriages between first cousins occur at a frequency of one to eight percent of all marriages, whereas amongst families showing rare metabolic anomalies such as alkaptonuria these marriages occur at about forty percent (Garrod 1931; also see Hogben 1939). This fact led Bateson and Saunders (1902), in the year in which the applicability of Mendel's factors to animals was being demonstrated by Bateson for the first time, to suggest that the trait was inherited as a simple Mendelian recessive. Hogben, Worrall and Zieve (1932), Hogben (1939) and Harris (1953) present discussions of the theoretical considerations that lie behind the genetic analysis of such rare abnormalities in humans.

Because of the expectation that normal precursors of homogentisic acid when fed to alkaptonurics would result in an increase of this acid in the urine, many series of compounds have been fed to these patients and to normal individuals serving as controls. In this manner, it was found that alkaptonurics when fed phenylalanine or tyrosine excreted more homogentisic acid in their urine, while none appeared in the urine of the controls. Thus, phenylalanine

and tyrosine are considered to be the initial precursors of homogentisic acid in man. The objection that homogentisic acid was a product of a secondary metabolism of phenylalanine and tyrosine, produced in the intestines of certain individuals (but not others) by bacteria harboring there, was overcome when no such bacteria could be demonstrated from attempts to isolate them from fecal material.

It is known that normal individuals who take homogentisic acid by mouth metabolize the substance so that none appears in the urine (Falta 1904; Leaf and Neuberger 1948). Homogentisic acid has been shown (Ravdin and Crandall 1951; La Du and Greenberg 1951), in rats, to be oxidatively cleaved between carbons 3 and 8 to yield 4-fumaryl acetoacetic acid (reaction 7, Fig. 1) which, in turn, is hydrolyzed by the addition of a molecule of water to fumaric acid plus acetoacetic acid (reaction 8, Fig. 1). These latter compounds can be further degraded in the organism to carbon dioxide and water (see review of recent isotope work on the catabolism of tyrosine by Greenberg 1954a). It is further known that 2,5-dihydroxybenzoic acid (gentisic acid) is excreted in the urine of alkaptonurics fed the compound, but not by normal individuals (Neubauer and Falta 1904). Benzoic acid, oxidized at any other positions than 2 and 5 in the ring, can be metabolized both by normal persons and alkaptonurics, indicating that the specific block is an inability to break the aromatic ring

structure of 2,5-dihydroxyphenyl compounds by those suffering from the defect. As long ago as 1914 the German biochemist, O. Gross, showed that the accumulation of homogentisic acid in alkaptonurics is, in some manner, due to a failure of enzyme activity. He found that homogentisic acid was degraded when placed in blood serum from normal individuals, but not when placed with serum from alkaptonurics. Recently, the Japanese workers Suda and Takeda (1950, cited by Greenberg, op. cit.) have reported an enzyme preparation from rabbit liver which they have named "homogentisicase". It appears that this homogentisic oxidase is necessary for the rupture of the benzene ring of homogentisic acid. It remains to be seen whether or not this enzyme is lacking in alkaptonurics but present in normal individuals.

Whatever the precise chemical mechanisms in the normal degradation of phenylalanine-tyrosine, Garrod as long ago as 1923 was able to make very clear that homogentisic acid was an intermediary compound in the catabolism of these two substances in man. Also he was able to state that a lack of enzymatic activity for the catabolism of phenylalanine and tyrosine, in individuals homozygous for the mutant gene concerned, resulted in an accumulation of homogentisic acid. This latter substance was then eliminated from the body in the urine. Curiously, these fundamental concepts in biochemical genetics went largely unnoticed by most geneticists for many

years. According to Neel and Schull (1954), "(a)lcaptonuria is ... not only the first recessive to be reported in man but the first biochemical mutant to be recognized in any species.". The pioneer role played by Garrod was first brought to the attention of geneticists by Hogben et al (1932), Hogben (1939) and Haldane (1935, 1937 and 1942), and has led Beadle (1951) to state: "It is proper that he should be recognized as the father of chemical genetics.".

Metabolic phenomena similar in nature to alkaptonuria are now known to occur in man (see Harris 1953 for an extensive review of the work in human biochemical genetics since the time of Garrod), several of which involve phenylalanine-tyrosine metabolism. Briefly, these latter are: 1. "albinism" (representing a block at reaction 3, Fig. 1) long known but first described in biochemical and genetic terms by Garrod (1902 and 1909), 2. "tyrosinosis" or "tyroketonuria" (representing a block at reaction 9, Fig. 1) described by Medes (1932) and 3. "phenylketonuria" or "phenylpyruvic oligophrenia" (representing a block at reaction 2, Fig. 1) first described by Fölling (1934). With the exception of tyroketonuria of which only a single case is known and a few cases of dominant inheritance of alkaptonuria which have been reported (see Harris 1953), the remainder of all cases studied having sufficient family data are inherited as autosomal recessives from usually normal parents heterozygous for the

gene. It is obvious that further discoveries of abnormalities involving phenylalanine-tyrosine metabolism in man would greatly extend, and perhaps modify, our existing knowledge of the biochemical reactions entered into by these two compounds *in vivo*.

With the observation of non-autonomous differentiation of vermilion eye color in mosaics of *Drosophila melanogaster* and *D. simulans* by Sturtevant (1920 and 1932), the way was open for an interesting series of analyses to be done on biochemical pathways in this organism (see Sturtevant 1932 for a rationale of this work; also see Ephrussi 1942 and Beadle 1945 for reviews). The actual proof of hormonal influences in insect eye color inheritance, suggested by Sturtevant, was initiated by Ephrussi and Beadle beginning in 1935 with the introduction of a technique for the transplantation of imaginal optic disc tissue from larvae on one genotype to the haemocoels of larvae of a differing genotype (Ephrussi and Beadle 1936). The technique was a modification of that used by Caspari (1933) in studying an eye color mutant of the meal moth, *Ephestia kühniella*. Historically, the onset of the *Ephestia* work antedates that begun with *Drosophila*. In *Ephestia kühniella* red eye color (*aa*) is recessive to black (*AA* or *Aa*). It was found that if a testis from an *AA* or *Aa* moth larva was transplanted into an *aa* larva, the latter animal developed black eyes instead of red as expected

(Caspari, op. cit.). This result was interpreted to mean that a chemical substance was present in the transplanted tissue which was necessary for the elaboration of black eye pigment. Because the substance was diffusible and separable from the tissue by alcohol and acetone, it was called a^+ hormone (see review of Ephestia work by Caspari 1949).

As many more eye color mutants are known in Drosophila than in Ephestia, transplantation experiments were greatly extended in the former. A somewhat similar situation developed when the eye color mutants, v (vermilion) and cn (cinnabar), were subjected to a corresponding analysis in this organism. Imaginal eye tissue anlage of v/v larvae when transplanted into wild-type (v^+/v^+) larvae developed into supplementary eyes usually in the abdominal cavity of the host. Pigmentation of the transplanted eye developed normally and showed that a production of brown pigment, formerly called "ommatin" but recently renamed "insectorubin", had taken place in this genetically constituted vermilion tissue (Ephrussi and Beadle 1935; Beadle and Ephrussi 1936). This again indicated the presence of a diffusible substance necessary for the formation of eye color pigment, and was called v^+ hormone. It was assumed that flies, homozygous for the vermilion allele, were deficient in this substance. Under the influence of the wild-type allelomorph of vermilion this substance is normally produced and is diffusible throughout

the body of the insect. The v^+ hormone then, much like homogentisic acid in alkaptonuria, represents a biochemical link between the gene and its phenotype.

Subsequent work, using a number of eye color mutations, showed that transplants of imaginal eye disc tissue from cn/cn larvae into wild-type larvae also developed non-autonomously. But all of the many other eye color mutants in Drosophila tested developed autonomously in such transplants (Beadle and Ephrussi, op. cit.). The question whether the v^+ and cn^+ cases involve the same substance or not was resolved by suitable reciprocal transplanatations between the two mutants. From the results obtained with these experiments, it was concluded that two substances were actually involved; the second becoming known as cn^+ hormone. It was further noted that a deficiency in the cn^+ hormone affected the production of eye pigment only in certain genotypes, whereas a deficiency in the v^+ hormone always resulted in a deficiency of the cn^+ hormone. Thus, it was postulated that the production of these two substances followed a metabolic sequence from v^+ to cn^+ (Beadle and Ephrussi, op. cit.).

The chemical identification of the v^+ hormone followed a series of diverse experiments performed by several investigators. Feeding experiments in which various substances, such as peptones of different origins, were incorporated into synthetic media led to the belief that the aromatic amino acid,

tryptophan, was a dietary precursor of the eye pigment (Khouvine, Ephrussi and Chevais 1938). Beadle and Law (1938) showed that crushed and boiled wild-type pupae fed to vermilion and cinnabar mutants would modify the eye colors of these mutants towards brown. Tatum (1939a) was able to show that bacteria, present in a single culture of a feeding experiment he was then performing, were responsible for the conversion of tryptophan to a v^+ hormone-like substance. Later, Tatum and Beadle (1940) succeeded in crystallizing this compound and ascertaining its empirical formula. With the knowledge that rabbits fed high levels of l-tryptophan excreted in their urine a substance, kynurenine, whose chemical structure was known, the German workers, Butenandt, Weidel and Becker (1940), tested the ability of kynurenine to replace the v^+ substance in both feeding and injection experiments. Of several compounds so tested, kynurenine gave positive results, indicating quite probably that kynurenine and v^+ hormone were identical. Later, Tatum and Haagen-Smit (1941) were able to show that the crystalline bacterial product of tryptophan metabolism was a sucrose ester of kynurenine, thus adding considerable support to the supposed identification. Final proof of this identity came with Kikkawa's experiments published in 1941. He found kynurenine actually accumulated in cn/cn Drosophila as expected from the interpretation given for the results of the transplantation

experiments. Kynurenine was not found in v/v larvae or pupae, a result similarly expected from previous work. Eight years later, Green (1949) found free tryptophan to be accumulated in vermilion Drosophila.

The cn$^+$ hormone proved even more difficult to identify chemically. Eventually, Butenandt, Weidel and Schlossberger (1949) isolated an oxidation product of kynurenine, 3-hydroxykynurenine, from the bluebottle fly, Calliphora, and found that it possessed cn$^+$ hormone activity for Drosophila.

For a considerable time alpha-hydroxytryptophan was considered to be an intermediate compound between tryptophan and kynurenine. It is known that in rat liver the conversion of tryptophan to kynurenine occurs in a step-wise fashion involving two intermediates of which the second is known to be formylkynurenine (Knox and Mehler 1950; Mehler and Knox 1950). Recently, Kikkawa (1953) has reported that alpha-hydroxytryptophan has no activity in Drosophila, but that formylkynurenine is effective in vermilion mutants. Thus, a series of step-wise biochemical reactions in the metabolism of dietary tryptophan which are under gene control in Drosophila can now be made out from a study of vermilion and cinnabar mutants. Tryptophan is believed to be converted (through at least one intermediate) to formylkynurenine. It is this conversion which is believed to be blocked in v/v mutants (see Figure 2, reaction 10). Formylkynurenine, in turn, is con-

verted to kynurenine (reaction 11, Fig. 2) and kynurenine is oxidized to 3-hydroxykynurenine (reaction 12, Fig. 2). 3-hydroxykynurenine eventually becomes elaborated as insectorubin through a series of unknown chemical intermediates (probably by way of quinone formation and linkage to a protein carrier). The eye color mutants st (scarlet) and cd (cardinal) seem to be involved at some stage along this route from 3-hydroxykynurenine to insectorubin. They both reduce the amount of insectorubin formed in the eyes, but as they develop autonomously it has been impossible to subject them to analyses similar to those performed with vermilion and cinnabar.

Mutations in different organisms can cause disturbances of metabolism similar to those demonstrated for vermilion and cinnabar mutants of Drosophila melanogaster. Thus, parallel eye color mutations in many insects have been proven to be disruptions of biochemical reactions like those found in D. melanogaster. A few of these cases are mentioned in the following paragraph.

First, among the Drosophila group (order Diptera), v^3 (vermilion-3) of D. virilis (Howland, Glancy and Sonnenblick 1937), v^{40^d} (vermilion-40^d) (Green 1952), v^{48^a} (vermilion-48^a) and cd (cardinal) of this same species (Price 1949) and v (vermilion) of D. simulans (Howland et al, op. cit.) all are unable to form kynurenine from tryptophan and are in this sense parallel to v in D. melanogaster. The mutants st

(scarlet) in D. virilis (Price, op. cit.) and or (orange) in D. pseudoobscura (Tan and Poulson 1937) correspond to cn mutants of D. melanogaster in being unable to convert kynurenine to 3-hydroxykynurenine. The cn (cinnabar) mutant of D. virilis develops autonomously and is thus considered to be parallel to st and cd in D. melanogaster. Second, among the bee and wasp group (order Hymenoptera), s (snow) of the honeybee, Apis mellifera, (Green 1955), parallels v in D. melanogaster; and i (ivory) in A. mellifera, o (orange) and o^{i} (ivory) in the parasitic wasp, Habrobracon juglandis (Beadle, Anderson and Maxwell 1938), correspond to the cn mutant of D. melanogaster. Third, among the moths (order Lepidoptera), a (red eye) of Ephestia kühniella (Caspari 1949) proved to be a parallel mutant of v in D. melanogaster, and w-1 (white-1) of the silkworm, Bombyx mori (Kikkawa 1941), parallels cn in D. melanogaster. White-2 in the silkworm develops autonomously and so corresponds to st and cd in D. melanogaster and to cn in D. virilis. The white-2 silkworms have been found to accumulate 3-hydroxykynurenine adding further support to the thesis that this mutant is blocked after 3-hydroxykynurenine formation from kynurenine (Kikkawa 1953).

Interesting chromosomal and genic relationships between different species within the Drosophila group have come to light as a result of analyses of mutations known to occur in them (see Sturtevant and Novitski 1941). Where possible, a

study of allelism among hybrids is made; where not, reliance is placed on careful studies of the similarity of gene effects within separate species. In five species of Drosophila these effects have been analyzed on a biochemical level and found to be blocks in similar biochemical reactions if not actually the same reaction. The species D. affinis, melanogaster, pseudoobscura, simulans and virilis all possess a gene on their X-chromosome whose mutant allele gives a vermilion eye color and causes a failure of kynurenine formation when present in the homozygous condition. Similarly, four of the above species possess a mutant gene on chromosomes homologous to the right arm of the 2nd chromosome of D. melanogaster whose effect is a cardinal eye color and a failure to produce 3-hydroxykynurenine. The exceptional species is D. simulans. The establishment of definite chromosomal and genic homologies between Drosophila and other insect groups have not been possible, of course, due to the gross differences in genetic constitution between the ordinal groups. Many authors, however, have speculated on the evolutionary significance of suspected homologies to the general problem of speciation (see Dobzhansky 1941).

Out of a vast number of possible examples, the final biochemical and genetic evidence to be reviewed briefly here was selected because it also involves inherited metabolic errors at specific points along the biochemical pathways entered

into by aromatic amino acids. This concerns, primarily, research performed with the filamentous fungus, Neurospora. Historically, the initiation of work with Neurospora followed a major portion of the investigations on the biochemical genetics of insect eye colors already described. On Beadle's own account (1951), it was frustration over the chemical identity of the cn^+ hormone in D. melanogaster that led to a search for a more suitable organism with which to do both biochemical and genetic research. As is well known, the heterothallic ascomycetes, Neurospora crassa and N. sitophila, were chosen (Beadle and Tatum 1941) since the life cycle of the mold had already been described (by Dodge in 1927) and its suitability for genetic research demonstrated by Lindegren (1932a, 1932b and 1933). In the succeeding 14 years this genus has proven to have distinct advantages over the organisms, maize and Drosophila, made classical in science by geneticists, and bacteria and man, studied so extensively by biochemists.

The synthetic capacities of Neurospora are of a high order. It is able to synthesize all of its protoplasmic constituents - proteins, carbohydrates, lipids, vitamins and nucleic acids - from a carbon source (as sugar), a nitrogen source (as nitrate), inorganic elements and one B vitamin - biotin - as starting material. It exists in two strains of different mating type so that mutagenically treated multi-

nucleated spores can be crossed with the untreated opposite mating type and segregation of the mutant nuclei followed. All four chromosomes of each tetraploid sporogonium segregate in regular order and in duplicate in the ascospore. In its vegetative stage it is haploid so that no problem of dominance occurs.

By a succession of nutritional tests, first on a "complete" medium consisting of yeast or malt extract and casein hydrolysate to supply additional growth factors, followed by single spore isolations onto "minimal" media, which require all the synthetic ability that the strains possess in order for growth to occur, the mutant forms may be distinguished from the wild-type by their failure to grow. By further single spore isolations onto various media, each containing one chemically defined protoplasmic component, the specific synthetic inability of the mutants can be identified. Several variations of this basic technique have been used with Neurospora and other microorganisms and will not be described here (see Tatum 1949 for a brief review of these methods).

Using Neurospora, an unexpected parallel was found between tryptophan metabolism in insects and the mold. It was found that Neurospora produces nicotinic acid, a vitamin necessary for the energy-metabolism of cells, from tryptophan (Beadle, Mitchell and Nyc 1947) and that the initial steps in

the conversion of tryptophan were the same in both organisms (Mitchell and Nyc 1948; Bonner 1948). Thus, tryptophan is converted to kynurenine through formylkynurenine and thence to 3-hydroxykynurenine in Neurospora as well as Drosophila; each step in the pathway was under separate gene control as shown by the proper genetic crosses. Parenthetically, it is interesting to speculate on the apparent genic homologies between these Neurospora genes and those controlling the same or similar reactions in Drosophila. But one should be cautioned concerning our ignorance of the actual gene action involved in each case before proceeding too far along these lines. From 3-hydroxykynurenine the pathways in the two organisms diverge. In Neurospora, 3-hydroxykynurenine is converted to 3-hydroxyanthranilic acid (see Figure 2, reaction 13) which, in turn, is converted to nicotinic acid through quinolinic acid (reactions 14 and 15, Fig. 2).

There are a number of different mutant strains known which require either tryptophan or nicotinic acid for growth. Strain C-83 cannot couple the aliphatic amino acid, serine, with indole (see Figure 1, reaction 16) to form tryptophan (Tatum and Bonner 1944; Mitchell and Lein 1948). Tryptophan or nicotinic acid will support the growth of this strain. Strain Y-31881 cannot utilize tryptophan but 3-hydroxy-anthranilic acid will support growth (reaction 13, Fig. 2). Strain 4540 accumulates the latter compound but growth occurs

when quinolinic acid is supplied exogenously. Quinolinic acid accumulates in strain 3416 (Bonner and Yanofsky 1949). This strain will grow only if nicotinic acid is supplied.

There are a number of other interesting mutants of Neurospora which involve metabolic blocks at various points preceding tryptophan synthesis. These will not be discussed here in any detail but are indicated in Figure 1 as reactions 17 to 21 (see Tatum 1951; Tatum, Gross, Ehrensvärd and Garnjobst 1954; and Barratt, Newmeyer, Perkins and Garnjobst 1954 for information concerning these mutants and the reactions which they control).

The One Gene - One Primary Function Hypothesis

The ready production of nutritional mutants by mutagenic agents in Neurospora and their subsequent genetic analysis, indicating that each biochemical reaction blocked is due to the presence of a single mutant gene, revealed a vast number of intermediate metabolic processes long suspected of occurring within cells. Further work with many other species of microorganisms has added more knowledge concerning these reactions. While the interests of the comparative biochemists were centered on these processes, many geneticists, struck by the one-to-one relationship between the mutated gene and the reaction blocked, renewed the attack on the old problem of gene action.

This one-to-one relationship was formulated by Beadle (1945) in the following terms.

"Self-duplication - In order to exist as such, genes must be capable of inducing the formation of exact copies of themselves. The way in which such self-duplication occurs is not known but is presumed to involve some type of model-copy mechanism.

"Heterocatalysis - In addition to catalyzing formation of more units like themselves, genes in general have heterocatalytic properties, that is, they catalyze the formation of other substances. The hetero- and auto-catalytic functions are probably essentially similar and consist of imposing specific configuration on protein or other molecules in the final step of their synthesis.

"Relation to specific chemical reactions - In determining the specific chemical and perhaps physical configurations of protein molecules, genes directly determine enzyme specificities and thereby control in a primary way enzymatic syntheses and other chemical reactions in the organism."

The foregoing ideas first became popularly known as the "one gene - one enzyme" hypothesis, but later it was rendered in less specific terms to take into account other possibilities for primary gene product formation (Beadle 1951). It has now become known as the "one gene - one primary function" hypothesis.

In the earlier writings of many other authors, who have speculated on the role that genes play in the development of an organism, similar concepts are stated either implicitly or explicitly (e.g., Garrod 1902; Moore 1910; Wheldale 1916; Troland 1917; Goldschmidt 1916, 1927 and 1938; Morgan 1926; Plunkett 1926 and 1932; Wright 1934; Powsner 1935; Haldane 1935 and 1937; Grüneberg 1938; Waddington 1940; Muller 1941; and many others).

Inasmuch as most biochemical reactions are catalyzed by enzymes, and a genetic change at a specific locus in Neurospora always results in an inability to perform the same biochemical reaction, the implications are that such metabolic blocks result from a loss of a functional enzyme and that the genes, in some manner, are concerned with their formation. It has been suggested many times that since genes are not used up or destroyed during the development of an organism, they might act directly as enzymes themselves (see McIllwain 1946) or they might serve as templates for the production of enzymes as primary products (see Wright 1941 and 1945). Unfortunately, we know comparatively little about the chemistry of the proteins of which enzymes are constituted and even less of the chemistry of the proteins and nucleic acids of genes (for papers dealing with recent research on the chemical structure of desoxyribonucleic acid, see Chargaff, Zamenhof, Brawerman and Kerin 1950; Pauling and Corey 1953a and 1953b;

Watson and Crick 1953a, 1953b and 1953c; and Chargaff 1955). Interesting theoretical attempts have been made recently to correlate the DNA structure postulated by Watson and Crick with protein specificity (e.g., Gamow 1954; Schwartz 1955 and others; see also Demerec 1955, p. 17). The validity of these theories, of course, as well as the Watson and Crick DNA model itself, must await experimental verification before much more can be said concerning this direct approach to the problem of gene action.

In the absence of any direct chemical evidence for or against the one-to-one hypothesis, several criticisms have been made largely based on indirect evidence which indicates that more complexity is involved than the theory allows. Some of these criticisms have been answered, in part at least, to the satisfaction of the tenets of the theory. Others require further work before a satisfactory commitment can be made one way or the other. In any case, it has been stated that the idea is not to be considered a biological law, but only a useful general working hypothesis (in discussion following paper by Bonner 1946b).

It has been noted (Harris 1953) that a failure to reabsorb an intermediary compound in the human kidney results in the excretion of that intermediary in the urine and not in its further normal metabolism. Such cases of a malfunction of renal reabsorption in human beings are known, although, as

far as is known, none of the phenylalanine-tyrosine metabolic errors mentioned previously fall into this category. It has been pointed out that non-reabsorption itself, however, may be due to an enzymatically determined alteration of the cytoplasmic constituents in such a way as to alter the permeability of the renal cell membrane. Or the genetic change at a single locus responsible for the defect may be acting so as to change the permeability of the membrane through an actual change in a structural component of the membrane (Haldane 1954, p. 84). Until further work is done on such cases, it is impossible to say whether or not they represent definite exceptions to the one-to-one hypothesis.

Delbrück (in discussion following paper by Bonner 1946b) criticized the one-to-one thesis on the basis that the probability of finding any multifunctional genes with the screening techniques employed in Neurospora research would be too low to be detected. Horowitz (1950) and Horowitz and Leupold (1951) have replied to this serious criticism by calculating that the selection of unifunctional mutants is not sufficiently intense, by the screening methods used to isolate the mutants, to account for the high frequency of such mutants actually found.

A shift in emphasis from studies of intermediate biochemical pathways in Neurospora to studies of the nature of the metabolic blocks themselves has taken place in recent

years. Some of these studies, as pointed out below, appeared to negate the one-to-one hypothesis at first, but have since been shown not to be in any fundamental disagreement with it.

Mitchell and Lein (1948), investigating the C-83 mutant of Neurospora which utilizes tryptophan for growth but, as mentioned previously, cannot carry out the condensation of indole and serine to form tryptophan, found that crude cell-free preparations of wild-type mold contained the enzyme tryptophan desmolase which catalyzes the coupling in conjunction with pyridoxal phosphate serving as coenzyme (see Umbreit, Wood and Gunsalus 1946). In the mutant no tryptophan desmolase activity could be detected by analytical methods which permitted maximum activities of 0.2 to 0.3% of those found in active mold to be detected. Within the limits of the technique employed, these results present clear-cut evidence for the view that genetic change at a locus results in a loss of an enzyme from the metabolic system. An extension of this work by Yanofsky (1952a and 1952b) has shown that in a closely related tryptophan mutant (Y-1952) there exists a suppressor mutation at another locus which enables the mutant to grow in the absence of tryptophan. The enzyme system concerned with tryptophan synthesis is found in cell-free preparations from this double mutant. The suppressor mutation has no discernable effect when combined with C-83.

Wagner (1949), working with mutants incapable of synthe-

sizing pantothenic acid from β-alanine and pantoyl-lactone (see Wagner and Guirand 1948), was able to show that acetone powders prepared from both the mutant and the wild-type mycelia showed normal enzyme activity for the condensation reaction involved. This result was quite unexpected in view of previous experience (see above) and from a priori reasoning from the one-to-one hypothesis. It was suggested that this evidence could be attributable to the presence of enzyme inhibitors in the cells of the mutants (Wagner, op. cit.) or the activation of the inactive enzyme by the acetone treatment itself (Horowitz 1950). Wagner and Haddox (1951), in a further study of these mutants, have now shown that the enzyme system found operative in vitro is also functional in vivo under certain environmental conditions. In the presence of the vitamin, the mutants were cultured under conditions of strong aeration. The mutants were then found to be able to synthesize more pantothenic acid than is supplied exogenously or found in normally grown wild-type strains. The present interpretation of these results suggests the possibility that the metabolic block is not concerned with the condensation reaction of β-alanine and pantoyl-lactone directly, but with an energy-providing system which is coupled to this reaction (Wagner and Mitchell 1955).

At present, there are a number of cases in Neurospora which indicate that complete enzyme loss need not be the

consequence of genetic change. There are a few mutations known which exhibit a block in a metabolic reaction when a particular hydrogen ion concentration is present in the medium, but not when the pH is changed (Stokes, Foster and Woodward 1943; Bonner and Beadle 1946; and Strauss 1951). There are a number of other mutations known which act in a similar fashion when grown at different temperatures (Mitchell and Houlahan 1946a and 1946b). Bonner, Yanofsky and Partridge (1952; see also Bonner 1952) have demonstrated the occurrence of incomplete genetic blocks in which the enzymes concerned are not completely lacking or inactivated. The demonstration consisted of a series of experiments which evolved from the assumption that if isotope-containing compounds which immediately succeed a metabolic block are given to the mutants, isolation of that compound after growth has occurred should show a dilution in its isotopic content provided synthesis of the compound has taken place. They were able to show that no isotopic interchange took place and that no alternative metabolic pathways in the synthesis of the compound were present in the mutants tested. Dilution was observed in several tryptophan-requiring mutants, indicating that some synthesis of tryptophan was taking place although at a rate too low for normal growth. Interestingly enough, the C-83 tryptophan mutant showed no appreciable dilution effect, thereby confirming the results of Mitchell and Lein previously mentioned.

The C-83 strain represents a case of a total block in metabolism which is not entirely lethal in its effect. It is apparent that genetic change, in the other tryptophan mutants tested, produces the necessary enzyme but in an ineffective state for normal growth.

Cases of multigenic control of a single enzyme have been reported as evidence against the one-to-one hypothesis (see Lederberg 1951). It has already been pointed out, however, that many genes may be required to build the requisite polypeptide structure of an enzyme but only a single gene required to impart its final specificity (in discussion following paper by Bonner 1946b). Studies of adaptive enzyme formation in bacteria led Monod (in Lwoff 1946) to the belief that a single genetic change can affect several different enzymes. Later, however, he withdrew the statement and has now reported that, in the absence of any contradictory evidence, he accepts the hypothesis that each adaptive enzyme is under single gene control (Monod 1947). Work with the A series of alleles (anthocyanin color) in maize had led Laughnan (1948) to the conclusion that some of the alleles in the series must be considered to be controlling two reactions. The precise reactions involved are not known. This author suggested the possibility that the one-to-one thesis may not be violated in this case provided that these alleles prove to be closely linked discrete units rather than true alleles. In the

following seven years, Laughnan presented the first analysis of position pseudoallelism in maize with regard to this series of alleles (Laughnan 1949, 1950, 1952 and 1955).

With the important experimental work of McClintock (1951) and Stadler (1951) on maize, together with the research on position pseudoallelism by Lewis (for references see Lewis 1955), Green (for references see Green 1954) and McKendrick and Pontecorvo (1952) on Drosophila, and Roper (1950; and in Pontecorvo, Roper, Hemmons, MacDonald and Bufton 1953) on Aspergillus nidulans, Benzer (1955) on Escherichia coli and Demerec, Blomstrand and Demerec (1955) on Salmonella typhimurium, has all profoundly changed the older concepts of genes. Once expressed in terms of an analogy to "beads-on-a-string", closely linked genes separable by crossing-over have been shown by these studies not always to be completely discrete functional units on the chromosome as formerly believed, but rather to be, perhaps, interactive physiological units in the chromosome not yet completely defined. At present there are two contrasting interpretations of position pseudoallelism (see Lewis 1955). Whichever of the two proves correct, neither can be said to contradict, in essence, the one gene-one primary function thesis at the present time, since they both concern the definition of a gene and do not raise fundamental questions as to the mode of function of genes which are at all contradictory to the hypothesis.

It must be emphasized, however, that the one-to-one hypothesis has arisen from a study of mutant genes of various kinds. There is no evidence whatsoever at present that the hypothesis is applicable equally to the action of normal allelomorphs. The activity of the normal gene may be much broader than, and different from, that of any of its mutants (see Ives 1939). It is conceivable that a change in one radical of a gene molecule (e.g., a hydroxyl group) would produce a mutant effect that could be detected. Other radicals (such as carboxyl groups) of that gene molecule, however, perhaps not as labile as the hydroxyl to mutagenic agents because of their positions in the structural configuration of the molecule or because of the strength of their chemical bonding, or both, could still be operative in controlling a different metabolic system after the mutation had occurred. Without improved histochemical techniques or direct knowledge of the chemistry of the gene, it is difficult to see how this important question can be solved.

The work to be reported here was initiated in order to explore some of the problems of biochemical genetics mentioned earlier. It was felt that the application of a genetically well-known organism to the study of metabolic processes would contribute to our knowledge in this field and offer, perhaps, some evidence on the nutritional, if not biochemical, genetics

of a diploid bisexual animal. The use of Drosophila in this way has entailed the acceptance of certain disadvantages not usually encountered in work with microorganisms. Drosophila has extremely low synthetic abilities as compared with Neurospora; it requires at least 10 amino acids, 8 vitamins, sugar, sterol and salts already preformed in its diet in order to grow and develop. Thus, the production of nutritional mutations by mutagenic agents is impracticable in this organism as many requirements are already present. As is well known, Drosophila does not grow at a uniform rate, but undergoes bursts of rapid growth following the larval instars and undergoes complete metamorphosis to the adult fly following the larval period itself. These facts offer complexity in the measurement of growth in nutritional studies. Wagner and Mitchell (1948) have presented a method whereby growth of Drosophila larvae on sterile media may be measured as a function of the content of the enzyme, adenosine deaminase, contained within the larvae. This procedure, although necessarily admitting several assumptions concerning the relationship between growth and enzyme content, permits reproducible results. Coupled with the techniques of raising large numbers of larvae under sterile conditions to be described later in this paper, it might well prove a useful tool for more exacting studies on the growth of larvae on various synthetic media. Finally, a further disadvantage lies in the size of

the organism. Although of great advantage in classical genetic studies, it presents some difficulty in the technical procedures applied to it during the establishment of a series of experiments under aseptic conditions.

Drosophila Nutrition

Under natural conditions Drosophila live on live yeasts of various kinds that grow in association with other microorganisms on decaying organic matter (Wagner 1944). Under laboratory conditions, media which contain live yeasts are quite heterogeneous; day-to-day changes in food value occur in such cultures as some yeast strains are favored while others are not (Robertson and Sang 1944). The elucidation of the inter-specific interactions that take place between developing Drosophila larvae and growing yeasts are of considerable importance to studies of the exhibition of genes whose variability may be environmentally determined. Gordon and Sang (1941) were able to show that variations in the exhibition of the gene "antennaless" were under nutritional control. Direct approaches to the ecological aspects of the problem of interactions between these dual populations in single cultures have been made recently (Robertson and Sang, op. cit.; Sang 1949a, 1949b and 1949c; Sang, McDonald and Gordon 1949; and Sang 1950). An understanding of the complete nutritional requirements of Drosophila might well aid in an

understanding of how specific dietary components might modify the exhibition of some genes as well as provide information on primary gene action.

Inherent in the development of knowledge of nutritional requirements of any organism is the concomitant achievement of axenic conditions. (The term "axenic" is here adopted, as suggested by Baker and Ferguson (1942) to replace such imprecise and awkward terms as "bacteria-free", "free-from-microorganisms", "sterile" etc. generally used in the literature. The word has been defined by the above authors (p. 116) to mean: "an axenic organism ... is a species free from any life apart from that produced by its own protoplasm.".)

In 1885, Louis Pasteur, in speaking of the possibility of life without the association of microorganisms always found in the alimentary tracts of animals, said: "Sans vouloir rien affirmer, je ne cache pas que j'entreprendrais cette étude, si j'en avais le temps, avec la pensée préconçue que la vie, dans ces conditions, deviendrait impossible.". The following year, however, Nencki (1886) pointed out that various digestive enzymes of the pancreas, stomach and intestine were capable of breaking down food into utilizable end-products without bacterial aid, whereas bacterial products themselves often contain substances harmful to the host organism. Although he performed no experiments himself, Nencki predicted success whenever such attempts were made.

Axenic Drosophila melanogaster (ampelophila Löw) were grown first by Delcourt and Guyénot in 1911. By repeatedly transferring a female fly from one aseptic bottle to another and allowing time for a few eggs to be deposited in each culture, these authors were able to keep a strain of flies for more than forty generations under axenic conditions. Guyénot (1913) used this strain for a series of studies on adaptation and fecundity under different environmental conditions. During the course of this work, he observed that dead yeast in aseptic media would completely replace live yeast as the principal food for the organism. As far as is known, this is the first reference to yeast as the chief component of the diet of Drosophila. For a number of differing reasons, several investigators since Guyénot's work was published have attempted to discover the factors contained in yeast which are essential to the animal. Much of this work has had to await the discovery and chemical clarification of one amino acid (threonine in 1935) and of the vitamins before progress could be made.

Loeb (1915a and 1915b) reported that Drosophila would grow and develop on a medium containing only inorganic salts, sugar and ammonium tartrate. At the time he believed that the synthetic capacities of the fly might be as great as those of bacteria. Later, he reported that most of the nutriment which the insects received came from the microorganisms present in

his cultures (Loeb and Northrop 1916). He then confirmed Guyénot's observations that yeasts go to make up the principal food of Drosophila. In these later experiments, the eggs of the fly were collected and then sterilized by immersion in 1% $HgCl_2$ in alcohol for six or seven minutes. In 1917, Northrop showed that a given quantity of yeast was capable of supporting the development of more flies when sugar, banana or casein was added to it. At about the same time, Baumberger (1917a, 1917b and especially 1919) and Baumberger and Glaser (1917), in a series of elaborate experiments, showed that yeasts were the major component of the insect's diet, that living yeasts were not present inside the eggs or in pupae formed from axenic larvae and that Drosophila can be conveniently reared on a solid agar medium supplemented with dead yeast. Baumberger, in 1919, also reported experiments involving extracted yeast nucleoproteins. No pupation was achieved by larvae grown on nucleoprotein alone, but excellent pupation ensued when sugars and salts were added to the medium in addition to the nucleoprotein. He was therefore able to show that sugar was essential in the diet, and that the nucleoproteins from yeast contained all the remaining indispensable substances.

Using aseptic techniques throughout their experiments, Bacot and Harden (1922) concluded from experiments using wheat-germ extract, yeast extract, butter-fat and lemon juice

that the complete development of the fly requires that vitamin B, but not vitamin C, be present in the medium. Thirteen years later, after the discovery that vitamin B contained more than a single growth factor, van't Hoog (1935a) was able to demonstrate that thiamine (B_1, aneurine) and riboflavin (B_2, lactoflavin) were indispensable to Drosophila. He could find no need for vitamins A, D or E by the developing organism. van't Hoog (1935b) was also able to detect traces of B_1 and B_2 (down to about 0.06 γ for B_1 and upwards from 1 γ for B_2) in crude materials fed to the larvae. He further found that a small quantity of Arachis oil, butter-fat, olive oil or similar products were essential to Drosophila.

The following year, van't Hoog (1936) reported that the indispensable fat fraction in the medium could be replaced by cholesterol. In addition, he tested several chemicals similar in structure to cholesterol and found cholesteryl-aniline, phenylcholesterol, phenylcholesterol-thio-ether, chlorcholestane and epidihydrocholesterol completely inactive in promoting growth and development. Dihydrocholesterol, however, proved to be active. He concluded that Drosophila shows a "sensitiveness" to stereoisomerization, by its ability to utilize dihydrocholesterol but not epidihydrocholesterol, similar to that shown by mammals to cholesterol and sitosterol.

The French investigator, Lafon, confirmed the observations of Bacot and Harden and van't Hoog that Drosophila does

not require vitamins A, C, D or E (Lafon 1937), and later showed that casein hydrolysate supplemented with tryptophan and cystine would supply the nitrogenous requirements of the fly (Lafon 1938). He concluded that there is an unidentified amino acid that is also essential to Drosophila since no growth occurred on a mixture of known free amino acids replacing the hydrolysate of casein.

By 1939, Tatum was able to add another vitamin of the B complex to the growing list of indispensable compounds required by the organism; this was nicotinic acid. He also was able to show that at least three unidentified growth factors were present in the extracts and residue of fractionated yeast. Fraction II (water-alcohol soluble fraction) combined with Fraction III (water soluble fraction) gave slow but otherwise normal development. Either of these fractions combined with Fraction I (water-alcohol insoluble residue) permitted pupation but did not promote eclosure. Completely normal growth and development did not take place unless all three fractions were added together to the basal medium then in use (Tatum 1939b). Subsequently, he showed that pyridoxine and pantothenic acid were the growth factors present in the water-alcohol soluble Fraction II (Tatum 1941).

In 1946, Schultz, St. Lawrence and Newmeyer announced in an abstract the successful achievement of complete growth and development of Drosophila in a synthetic medium all of whose

components (except agar) were chemically defined when a mixture of amino acids was used in place of casein hydrolysate. The water soluble Fraction III of yeast was replaced by biotin, folic acid and ribonucleic acid. Other B vitamin-complex constituents added to the medium, not previously reported as being used by other workers, were choline and para-aminobenzoic acid. The achievement of complete growth and development clearly indicated that all indispensable substances for Drosophila were known and present in the medium. Pupation, however, was reached in 8 or 9 days at 25°C, some 4 days slower than that obtained when the larvae are fed on yeast at the same temperature. Experiments with varying concentrations of vitamins and different protein levels led these authors to the conclusion that an unidentified growth stimulatory substance was present in the insoluble portion of yeast. Deoxyribonucleic acid was reported as retarding the development of the larvae, although ribonucleic acid stimulated development considerably.

Villee and Bissell (1948) investigated the role of nucleic acids in Drosophila nutrition and claimed that the ribotides of RNA were as effective in promoting growth and development as the whole molecule of RNA. These authors also claimed that the stimulatory property of RNA lay in the purines and pyrimidines (especially adenine) rather than in RNA itself. They found that the addition of adenine to a medium otherwise lack-

ing in RNA or its components or derivatives accelerated growth over that obtained with media containing RNA. These results are at variance with those of Schultz et al (1946) who reported that the ribotide, adenylic acid, could substitute "to a degree" for RNA, but that the purine and pyrimidine bases could not. The present author found, using a wild-type strain (Oregon-R), that none of the purine or pyrimidine bases, singly or in combination, could substitute for the whole RNA molecule (Ellis 1950). Sang (personal communication 1955), in a preliminary investigation using Oregon-S, has also found that none of the natural or derived purine or pyrimidine bases substitute for RNA when tested singly. A larval developmental rate, equal to that obtained when RNA is supplied in the medium, was achieved by the addition of the two ribotides, adenylic and cytidylic acids, in place of RNA. Either ribotide alone had no such stimulatory effect.

Villee and Bissell (op. cit.) further reported an inhibition of larval growth and pupation by benzimidazole, a compound chemically similar to the purines. Later, Villee and Lowens (1948) reported, in an abstract, studies of an inhibition of growth and pupation by barbituric acid, a pyrimidine compound. They found that RNA would release this inhibition, but adenine would not.

Hassett (1948), in a study mainly concerned with the utilization of various sugars by adult Drosophila, employed

axenic larvae on a dead yeast medium in one portion of his research. He tested three sugars singly in this medium and found that fructose gave better pupation and more adults than sucrose which, in turn, was better utilized than glucose.

Rudkin and Schultz (1947), in an abstract, reported that Drosophila require the same ten amino acids found indispensable for mammals, birds and the protozoan Tetrahymena. Tryptophan could not be shown to spare nicotinic acid (Schultz and Rudkin 1948) as might be expected if the metabolic pathway from tryptophan to nicotinic acid, found operative in Neurospora, were functional in Drosophila as well.

In experiments designed to elucidate the nature of the growth stimulatory substances still unidentified in the medium, Begg and Robertson (1948 and 1950) and Begg (1949) reported that they could find no need for a water insoluble yeast fraction as previously reported by Tatum (1939b) and Schultz et al (1946). They found all the factors necessary for normal development present in the water soluble yeast autolysate they obtained. They state, however, that this discrepancy may be due to the procedures of extraction employed rather than due to any fundamental differences in the growth stimulatory factor(s). Their alkali-soluble fraction of the yeast autolysate, which gave completely normal development in an otherwise synthetic medium, showed nucleoprotein-like reactions but was shown to be neither arginine nor

nucleic acid. These authors also confirmed the need for choline and folic acid by Drosophila originally reported by Schultz et al (1946). Begg (1949) reported a stimulatory effect of thymine and confirmed the observation of Schultz et al (1946) that DNA is inhibitory to development. Begg (1949; see also Begg and Robertson 1950) also found evidence that some specific amino acid or particular short polypeptide molecule is stimulatory to Drosophila development.

Hinton, Noyes and Ellis (1951; a reprint of the published paper is appended to this thesis as Appendix A), in an extensive study of amino acids and vitamins in a completely chemically defined medium (except agar), found that D-tryptophan was entirely inactive in larval nutrition while the L-isomer was utilized and was essential to the organism. High concentrations of tryptophan (6 mg/ml), however, produced multiple tumors and eye malformations (see Plaine and Glass 1955 for further work on tumor production using tryptophan, and for references). D-serine was found to be extremely toxic, and L-serine was slightly toxic under most conditions. Glycine in high concentrations had a detoxifying effect on the essential amino acids present in the medium. Vitamin B_{12}, thioctic acid (6,8-dithiooctanoic acid, protogen, lipoic acid), and B_T (carnitine) were found to have almost no discernable effect on growth and development. Inositol and para-aminobenzoic acid were shown to be unnecessary in the medium; PABA being

somewhat inhibitory. Biotin, pantothenic acid, choline, folic acid, nicotinic acid, riboflavin and thiamine were found to be essential to development of the larvae, thus confirming the work of several earlier workers (see above). Dose response experiments to pyridoxine gave inconsistent results and no conclusion was reached as to its indispensability to the organism. It was suggested that the inconsistencies encountered were due to contaminations of pyridoxine in the nucleic acids used.

Sprites (1951), in an abstract, reported that mature Drosophila larvae, like most organisms, contained the Krebs cycle enzymes, aconitase, isocitric dehydrogenase, succinic dehydrogenase, fumarase, malic dehydrogenase and condensing enzyme (condenses oxalacetic acid with "active acetyl" from pyruvic acid to form citric acid). These enzymes were demonstrated by spectrophometric and Warburg techniques on unwashed homogenates, water extracts and acetone powder extracts. Sprites (op. cit.) also reported the presence of cytochrome oxidase, oxalacetic decarboxylase and lactic dehydrogenase in mature larvae.

Recently, Hinton and Laszlo (in Fraenkel, Friedman, Hinton, Laszlo and Noland 1955) have reported that either natural L-carnitine or synthetic DL-carnitine are able to substitute completely for choline in the diet of Drosophila. This result differs from that obtained with tests on a

choline-less mutant of Neurospora crassa, the German cockroach, Blatella germanica, and the flour beetle, Palorus ratzeburgi, all of which failed to show any such replacement (Fraenkel et al, op. cit.).

In summary, Drosophila larvae can now be said to have as indispensable components of their diet the following:

1. As a source of nitrogen: The ten essential amino acids found indispensable also for birds, mammals and Tetrahymena (argenine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine).
2. As a source of carbon: sugar; fructose more utilizable than sucrose or glucose.
3. The sterol: cholesterol.
4. The vitamins: thiamine, riboflavin, pyridoxine, pantothenic acid, biotin, folic acid, choline and nicotinic acid.
5. Although not proven essential because of technical difficulties: inorganic elements as salts.

For normal development there still remain unidentified growth stimulatory substances one of which is nucleoprotein in nature, while another may be either a specific amino acid still to be identified or a short polypeptide molecule.

Kidder (1953) has recently reviewed the comparative nutritional requirements in all invertebrate animals tested

under axenic conditions. Therefore, these comparisons will not be described here. Since that review, however, a series of reports have appeared on nutritional research done under axenic conditions with another dipterous insect besides Aëdes and Drosophila, the two best known nutritionally. This organism is Pseudosarcophaga affinis (Fall.), a parasite of the spruce budworm, Choristoneura fumiferana (Clem.) (House 1954a, 1954b, 1954c and 1954d). These studies show that this insect possesses essentially similar nutritional requirements as Drosophila with the exception that no requirement could be found for folic acid and pyridoxine. This result is unusual since it is known that Drosophila and most other animals require folic acid and pyridoxine. It is apparent that small traces of these compounds could be present in the media as contaminants of other constituents. In any case a repetition of this interesting study of a parasitic form, after all possible sources of these vitamins have been eliminated from the components of the medium, would be most desirable.

More recently, Brust and Fraenkel (1955) have reported on the axenic culture of the blowfly, Phormia regina (Meig.), an insect known to harbor the poliomyelitis virus in areas where there is a high incidence of the disease. Although no study was made of amino acid requirements for this insect, the nutritional requirements for the vitamin and sterol components of the diet are similar for this fly as for Drosophila

melanogaster. The statement is made, however, that folic acid and choline are only required as stimulatory substances since some growth occurs in their absence. Regarding highly active folic acid, the same criticism given above for House's work may also apply here; namely that folic acid might be supplied to the organism as chemical contamination. On the other hand, it is possible that the newly emerged larva carries over sufficient folic acid from the egg to supply the need for this substance throughout its life. Requirements for choline vary in animals so that it is possible that Phormia differs from Drosophila with respect to a requirement for this vitamin. Contrary to what has been found for Drosophila, it is stated that RNA, DNA, adenine, guanine and uracil have no effect on growth for Phormia either when supplied singly or in combination. It is also stated, however, that eclosion is inhibited by the above compounds and DNA promotes pupation "supported" by RNA, although RNA alone has no such effect (Brust and Fraenkel, op. cit.).

A Ribonucleic Acid Requirement in a Strain of Drosophila

As first pointed out by Schultz and Rudkin (1949) there are four obvious ways in which a study of nutritional requirements in Drosophila can be used. These are: 1. nutritional differences between genotypes can be studied (see Rudkin and Schultz 1949; and Schultz and Service 1951); 2. variations of

specific components of the medium affecting the expression of variable genetic characters, as previously mentioned, can be investigated (see Gordon and Sang 1941); 3. comparisons of nutritional deficiency syndromes of wild-type flies which, as larvae, were grown on deficient media, and mutants grown on complete media can be made; and finally 4. nutritional mutants involving biochemical reactions of special interest can be studied, and their mode of inheritance from one generation to the next elucidated.

It should be emphasized again that the latter category above, at present, offers little hope of full exploitation on the Neurospora model due to the already complex nutritional requirements of the developing fly. A ribonucleic acid-requiring mutant of Drosophila was discovered, however, which has been of special interest to the author. The work preceding its discovery began with a series of studies by Hinton and Atwood (1941) and Hinton (1949a and 1950) on a strain of Drosophila melanogaster Meigen containing a second chromosome inversion. This strain was derived from a single female found among the F_1 of ri p^p (radius incompletus, peach) flies that had been irradiated with 4,000 r X-rays. The inversion, designated In (2LR)40d, contains a break in the euchromatic region 26D (Bridges' map) just before 26E in the left arm of the second chromosome, and a break in the heterochromatic region of the right arm of the same chromosome at 41 A/B.

The reversal of the segment of chromosome between the two breaks results in the euchromatic region 26D lying adjacent to the heterochromatic region 41B, and the euchromatic region 26E lying next to the heterochromatic 41A (see Hinton 1950 for further discussion of cytological analyses). The inversion, lethal when homozygous, has been carried over the "Curly" inversion (In (2L)Cy, In (2R)Cy) along with the recessive mutant gene sp^2 ($speck^2$).

In (2LR)40d/Cy sp^2 exhibits a disarrangement of the eye facets, mottling of the eye pigments and, occurring singly or in groups, dark patches of a "tumor-like substance on the surface of the eye" interspersed over and among the disarranged facets (Hinton 1947). It has been reported by Hinton (1948a, 1948b, 1949b and 1950) that the inversion produces this phenotypic expression only when the heterochromatic regions are in their new positions as described above. In one case, however, the phenotypic expression reverted to the wild-type with no corresponding positional change in the heterochromatic material that could be detected by salivary gland analysis (case IIDD; Hinton 1950).

The phenotype produced by In (2LR)40d is variable in expression among siblings raised under similar environmental conditions. Hinton (1949a), in a study of the production of modifications in the phenotype of In (2LR)40d, was able to show that: 1. the last adults to emerge in a culture showed

a more extreme effect than those emerging first; 2. larvae, raised under crowded conditions, were significantly more affected, as adults, than those raised under conditions where abundant food was available; 3. adults resulting from the last eggs laid by the mother were more extremely affected than those resulting from the first eggs laid; 4. offspring raised at 15°C and 28°C were less affected than those raised at the intermediate temperatures; 5. the presence of a Y-chromosome in the female reduced the effect as compared to their normal brothers. Age of the individual fly, age of the parents and sex had little or no influence on the phenotype.

The observations noted above were interpreted to mean that an environmental influence was affecting the production of the phenotype. That this influence might be nutritional was shown by theoretical considerations of items 1, 2, 3, and 4 above (see Hinton 1949a for details) all of which could be interpreted as indicating the possibility that a biochemical reaction of some sort was involved. Because the phenotype was known to be dependent upon the new close proximity of two euchromatic regions of the chromosome to heterochromatic regions following chromosomal breakage, it was reasoned that any environmental influences which alter the expression of the inversion would alter it first by affecting the heterochromatic regions involved in the breaks. Further, it was postulated that the heterochromatic regions inhibit the

expression of certain normal genes lying adjacent to them to produce the phenotypic effect.

Following the explanation for "mottling" effects presented by Schultz (1936), Schultz and Caspersson (1939), Schultz, Caspersson and Aquilonius (1940) and Schultz (1941a, 1941b and 1947) indicating that the juxtaposition of heterochromatic and euchromatic regions in the chromosome in new ways in some manner upsets the nucleic acid metabolish taking place at regions near the heterochromatic material, it appeared profitable to search for a nutritional enhancement or deficiency in ability to synthesize nucleic acid by the In (2LR)40d strain. (See discussion of Schultz's views in White 1945, p. 68, and a criticism of the ultraviolet technique (Commoner 1949) used by Caspersson and his co-workers.) It was felt at that time that a difference in synthetic capacity for nucleic acid in this inversion-containing strain might provide information concerning the underlying mechanism of the observed phenotypic expression, and eventually serve as a basis for a more detailed explanation of such "position effects".

Accordingly, techniques were devised to permit the raising of Drosophila under axenic conditions together with as completely a defined chemical medium as possible. The initial medium used was similar to that announced by Schultz et al (1946) and contained free amino acids as sources of nitrogen.

It permitted growth and development of the larvae through to the adult stage, although at an extremely slow rate as compared to the rate of growth obtained when larvae were grown in the presence of living yeasts. By the omission of nucleic acid (or any of its constituents or derivatives) from the diet, it was possible to show that In (2LR)40/Cy sp^2 larvae would not develop under these deficient conditions, whereas a wild-type strain (Oregon-R) would reach the adult stage in considerable numbers under the same dietary conditions (Hinton, Ellis and Noyes 1951; a copy of the published paper reporting these nutritional experiments is appended to this thesis as Appendix B). The purine base, adenine, or its riboside or ribotide were found to be effective in permitting growth of In (2LR)40d/Cy sp^2 larvae through to the emergence of adults in place of the entire ribonucleic acid molecule. While guanine and guanylic acid did support some growth of the inversion stock, none of the other nucleic acid components did so when tested singly or in any combination which lacked adenine. It was not felt that guanine or guanylic acid were replacing adenine in Drosophila nutrition, but rather that these compounds contained adenine as contamination. The riboside, guanosine, gave no growth when present alone in the basal medium.

It remained to determine whether or not the nutritional requirement for nucleic acid was, in any way, associated with

the interbrachial inversion of the second chromosome, and consequently of the phenotype produced. Recently, Hinton (1955) has reported that the nucleic acid requirement of In (2LR)40d/Cy sp^2 is associated with the second chromosome and not with any other chromosome. He suggests that the basis of the nucleic acid requirement "lies in the heterochromatin and is a property of the altered heterochromatin itself.".

Although so stated in Hinton, Ellis and Noyes (1951) that the RNA requirement represents the "first clear-cut case in animals of the inheritance of a basic biochemical difference involving a nutritional deficiency", it is clear, as pointed out by Wagner and Mitchell (1955), that a consideration of vermilion and cinnabar eye color mutants of Drosophila show that they too fall into the category of nutritional mutants, since they are able to utilize kynurenine and hydroxykynurenine in their diets to restore normal eye color. In general, however, eye color mutants of Drosophila do not reveal biochemical reactions which are of such fundamental importance to the organism as nucleic acid metabolism is in In (2LR)40d.

A second nucleic acid-requiring mutant of Drosophila was discovered by Sang (unpublished) using axenic media containing whole casein. This nutritional mutant is a wild-type strain, Oregon-K, which had been inbred, reputedly, for over 700

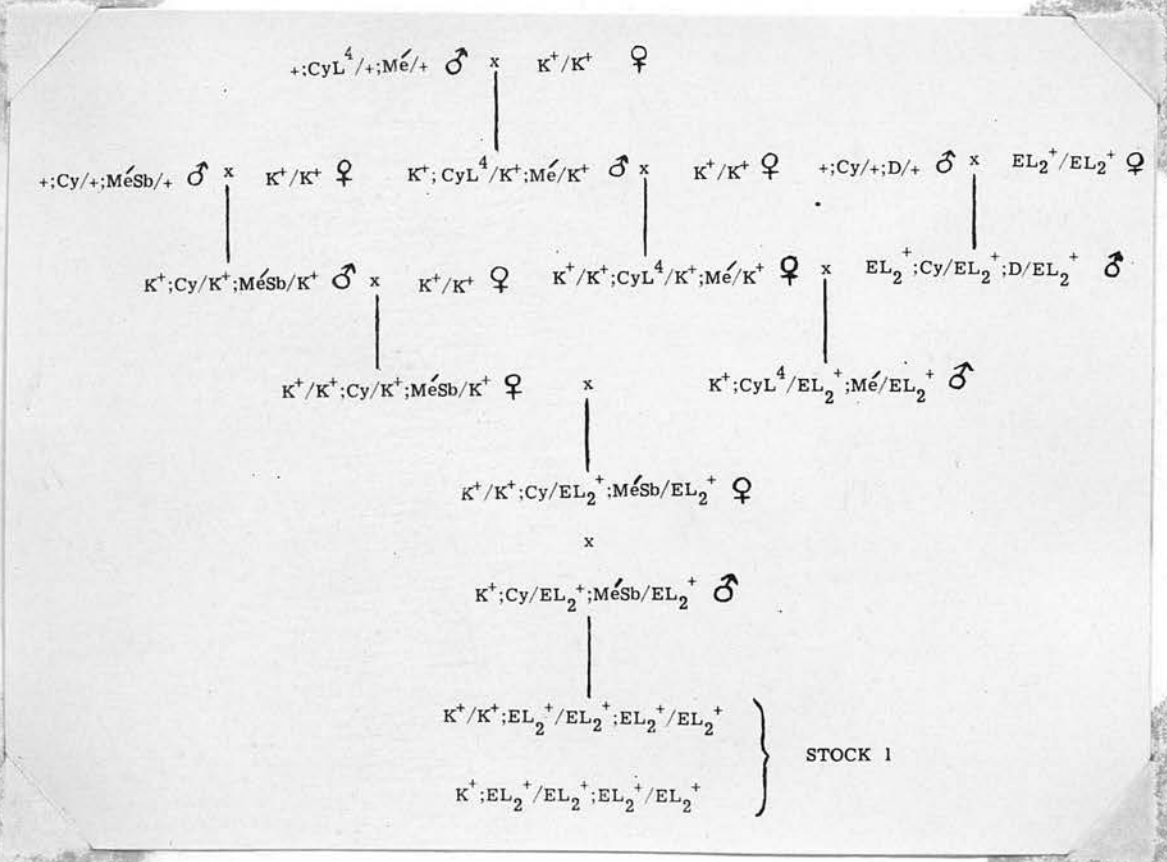
generations. Our work to be reported here was begun by an attempt to analyze this strain genetically in order to determine the mode of inheritance of a nutritional deficiency for nucleic acid. Our failure to achieve this initial objective (see Experimental and Discussion) led us to a search for a suitable genetically marked stock to be used in crosses with Oregon-K in a further attempt at genetic analysis. During the course of the nutritional study of one of the marker stocks tested, the discovery was made that the pH of the medium, under certain conditions, markedly affected both the rate of growth and the development of this stock. Subsequent work with Oregon-K and In (2LR)40d/Cy sp^2 larvae grown on nucleic acid-deficient media confirmed the observation of a pH effect on growth and development. This discovery represents the major contribution of the work to be presented here.

MATERIALS AND METHODS

The work to be reported here was performed in two places, some 3,000 miles apart; at the Institute of Animal Genetics, University of Edinburgh, Edinburgh, Scotland and at the Biological Laboratories, Amherst College, Amherst, Massachusetts USA.

Stocks Used: Several different mutant and wild-type stocks of Drosophila melanogaster were used in the course of this investigation. In one series of experiments a stock was made up which contained its X-chromosome(s) derived from a nucleic acid-requiring, phenotypically wild-type strain, K^+/K^+(Oregon-K), while the 2nd and 3rd chromosomes were derived from a non nucleic acid-requiring, phenotypically wild-type strain, EL_2^+/EL_2^+ (Edinburgh-large). The matings performed are given in Figure 3. They involved the use of stocks containing the genetically marked 2nd chromosomes, Cy (Curly) and L^4 ($Lobe^4$), and the genetically marked 3rd chromosomes, D (Dichaete), Mé (Moiré) and Sb (Stubble), in addition to the wild-type unmarked chromosomes mentioned above. Using similar matings, stocks were also made up which contained the 2nd and 3rd autosomes homozygous for the Oregon-K chromosomes; the remaining chromosomes (either 2nd, 3rd or X) in each stock were derived from Edinburgh-large. The stocks, then, were constituted as follows: K/K;EL/EL;EL/EL - EL/EL;K/K;

Figure 3



Scheme of matings used to obtain Stock 1 which was hemi- or homozygous for Oregon-K X-chromosome(s) with its second and third pairs of chromosomes derived from Edinburgh-large (EL_2). Similar matings were used to obtain Stocks 2 and 3 whose second and third chromosomes respectively were derived from Oregon-K.

EL/EL - EL/EL;EL/EL;K/K. They will be referred to simply as Stocks 1, 2 and 3 respectively.

A number of other Drosophila strains were used, both mutant and wild-type, for nutritional tests. These will be mentioned, together with the experiments in which they were used, in a later section of this thesis.

Media Preparation: Although several minor variations were made in the manner of preparing media throughout the duration of this work, in general two major methods were employed. One of these consisted of the preparation of media containing amino acids as nitrogen source; the other employed whole casein in place of the amino acids. The compositions and final concentrations of each ingredient in the various media used are given in Table 1 (amino acid media) and Table 2 (casein media). The concentrations are expressed in micrograms per milliliter (γ/ml) for the vitamins and milligrams per milliliter (mg/ml) for the remainder of the components of the media. This has been done to facilitate comparisons with other animal nutritional work. A dash in the column labeled "No. of Medium" indicates that that particular component of the medium was omitted. An "S" indicates the component was used in the same concentration as indicated in the preceding column(s). A third type of medium was used briefly for comparative purposes in a single series of experiments. This was a dehydrated medium prepared commercially by

TABLE 1

MEDIA USED CONTAINING AMINO ACIDS

Component	No. of Medium		
	1	2	3
	mg/ml.		
alanine	DL-1.085	S	-
arginine	L-0.794	L-0.559	L-0.794
aspartic acid	L-1.221	S	-
cysteine	L-0.480	S	S
glutamic acid	L-4.418	S	S
glycine	1.745	0.969	1.745
histidine	L-0.484	S	S
hydroxyproline	L-0.384	S	-
isoleucine	L-1.260	DL-1.260	L-1.260
leucine	L-2.345	S	S
lysine	L-1.337	S	S
methionine	DL-0.339	S	S
phenylalanine	L-1.008	S	S
proline	L-1.682	S	-
threonine	DL-0.756	S	S
tryptophan	L-1.745	DL-3.00	L-1.745
tyrosine	L-1.240	S	-
valine	DL-1.355	S	S

TABLE 1 (continued)

MEDIA USED CONTAINING AMINO ACIDS

Component	No. of Medium		
	1	2	3
	mg/ml.		
sucrose	7.5	S	S
cholesterol	0.1	0.4	0.1
ribonucleic acid	3.0	1.0	3.0
salts (Tatum 1939b):			
$MgSO_4 \cdot 7H_2O$	0.246	0.0129	0.246
$MnSO_4 \cdot 4H_2O$	0.0129	0.246	0.0129
$FeSO_4$	0.0129	0.129	0.0129
KH_2PO_4	0.606	S	S
K_2HPO_4	0.606	S	S
NaCl	0.0129	S	S
$CaCl_2$	0.0129	S	S
inosine	-	0.25	-
thymine	-	0.004	-
agar	15.0	S	S
	γ/ml.		
biotin	0.020	S	S
B_{12}	-	0.04	-

TABLE 1 (continued)

MEDIA USED CONTAINING AMINO ACIDS

Component	No. of Medium		
	1	2	3
	γ/ml.		
ca-pantothenate	6.0	S	S
choline chloride	20.0	S	S
niacinamide	10.0	S	S
pterylglutamic acid	6.0	S	S
pyridoxin	30.0	3.0	30.0
riboflavin	2.4	S	S
thiamine	1.5	S	S

TABLE 2

MEDIA USED CONTAINING WHOLE CASEIN

Component	No. of Medium		
	1	2	3
	mg/ml.		
whole casein	20.0	40.0	50.0
tryptophan	DL-1.0	S	-
fructose	-	10.0	-
sucrose	20.0	-	7.5
cholesterol	1.0	2.0	0.15
ergosterol	1.0	-	-
lecithin	-	-	2.0
ribonucleic acid	0.68	8.0	4.0
thymine	0.005	0.002	-
agar	20.0	22.0	25.0
$MgSO_4 \cdot 7H_2O$	0.09	0.20	0.246
$MnSO_4 \cdot 4H_2O$	0.04	0.02	0.0129
NaCl	-	0.02	0.0129
$CaCl_2$	0.04	0.02	0.0129
$FeSO_4$	0.05	0.02	0.0129
$NaHCO_3$	2.66	-	-
Na_2HPO_4) As Sorensen's buffer	-	3.04	S
KH_2PO_4) (pH 7.4)	-	0.71	S

TABLE 2 (continued)

MEDIA USED CONTAINING WHOLE CASEIN

Component	No. of Medium		
	1	2	3
	γ/ml.		
biotin	0.2	0.2	0.06
ca-pantothenate	2.0	2.0	3.0
choline chloride	20.0	40.0	S
inositol	40.0	-	-
niacinamide	10.0	S	1.0
paraminobenzoic acid	2.0	-	-
pterylglutamic acid	2.0	2.0	0.6
pyridoxine	2.0	2.0	0.4
riboflavin	2.0	2.0	S
thiamine	1.4	1.5	1.0

Difco Laboratories, Detroit, Michigan. Its composition was essentially similar to Medium No. 4 as reported by Hinton, Noyes and Ellis (1951; see Appendix A), but it contained no ergosterol, 0.4 mg/ml of cholesterol (instead of 0.1 mg/ml), and 0.04 γ/ml of vitamin B_{12} (instead of 0.028 γ/ml). Its composition is given in Table 1 under column 2.

1. Amino Acid Media: A measured amount of cholesterol dissolved in ether was pipetted into the bottom of each test tube to be used. Each amino acid (and sucrose) was weighed out on an analytical balance. The dry substances were then pooled in a mortar and ground to a fine powder. A measured amount of distilled water was added and the mixture neutralized with 1N NaOH. The remaining components of the medium, which had been previously made up in concentrated stock solutions (vitamins were made up fresh each week; other components, once per month) and stored in the cold under a layer of toluene, were added in the following order: nucleic acid (if used), the chlorides, the phosphates, the sulphates (ferrous sulphate solution was made up anew for each experiment) and the vitamins. The mixture was again neutralized and the final volume brought up with distilled water. A measured quantity of agar was added, and the solution brought to the boiling point on a water bath. After thorough mixing, a measured amount of the final medium was pipetted into each test tube. The tubes were plugged with nonabsorbent cotton

and autoclaved for 10 minutes at 15 lbs. pressure. Once autoclaved and after thorough shaking to mix the ingredients, they were placed on a slant (only for the 15 x 150 mm tubes; see below) and left to cool and solidify. The same procedure was employed when large boiling tubes were used except that they remained in an upright position while cooling. The tubes were stored in a refrigerator, after hardening, until ready for use. It was found that storage of prepared media for longer periods than one week significantly increased the time of larval development and decreased the yield of flies. Therefore, all media were used within a week of preparation and usually within 24 hours.

2. Casein Media: In the early experiments performed, measured amounts of casein, agar, sugar, tryptophan and cholesterol were weighed out and ground together in a mortar. Measured amounts of the dry mixture were then weighed and added to each boiling tube. The salts were pipetted into a beaker, followed by nucleic acid (if used) and the vitamins. This solution was brought up to final volume by the addition of distilled water containing enough sodium bicarbonate to neutralize the resultant medium. Measured amounts of the solution were added to each tube. These were stoppered with nonabsorbent cotton and autoclaved under pressure. Medium 1, Table 2 was made up in this manner.

In later experiments this method was modified in an

attempt to overcome the obvious concentrational discrepancies arising from the placement of measured amounts of a mixture of dry material into the boiling tubes. An example of the modification used is as follows: To prepare 400 ml of medium sufficient for 100 tubes containing 4 ml each, 20.0 gm of whole casein, 3.0 gm of sucrose and 10.0 gm of agar were weighed and ground to a fine powder. 0.06 gm of cholesterol and 0.4 gm of lecithin were weighed and placed in a beaker. Once these latter substances were dissolved in ether, they were added to the dry mixture and stirred until the ether had evaporated. 160 ml of Sorensen's buffer (pH 7.4) was added followed by 0.4 ml of chlorides, 8.0 ml of sulphates and 1.95 ml of vitamins added from stock solutions. The total volume of 170.35 ml at this stage was brought up to 300 ml by the addition of distilled water containing enough 1N NaOH to neutralize the solution. Hydrogen ion concentration readings were made with a Beckman glass electrode pH meter. At this point, the medium was divided up into equal portions and the variable (usually nucleic acid or its constituents) added to some of them. Final volumes were adjusted according to the proportional amount used to bring the total to 400 ml. The medium was pipetted into individual tubes which were plugged with cotton and autoclaved as previously described.

Stock Maintenance: Stocks of flies to be tested nutritionally were grown in half-pint milk bottles containing

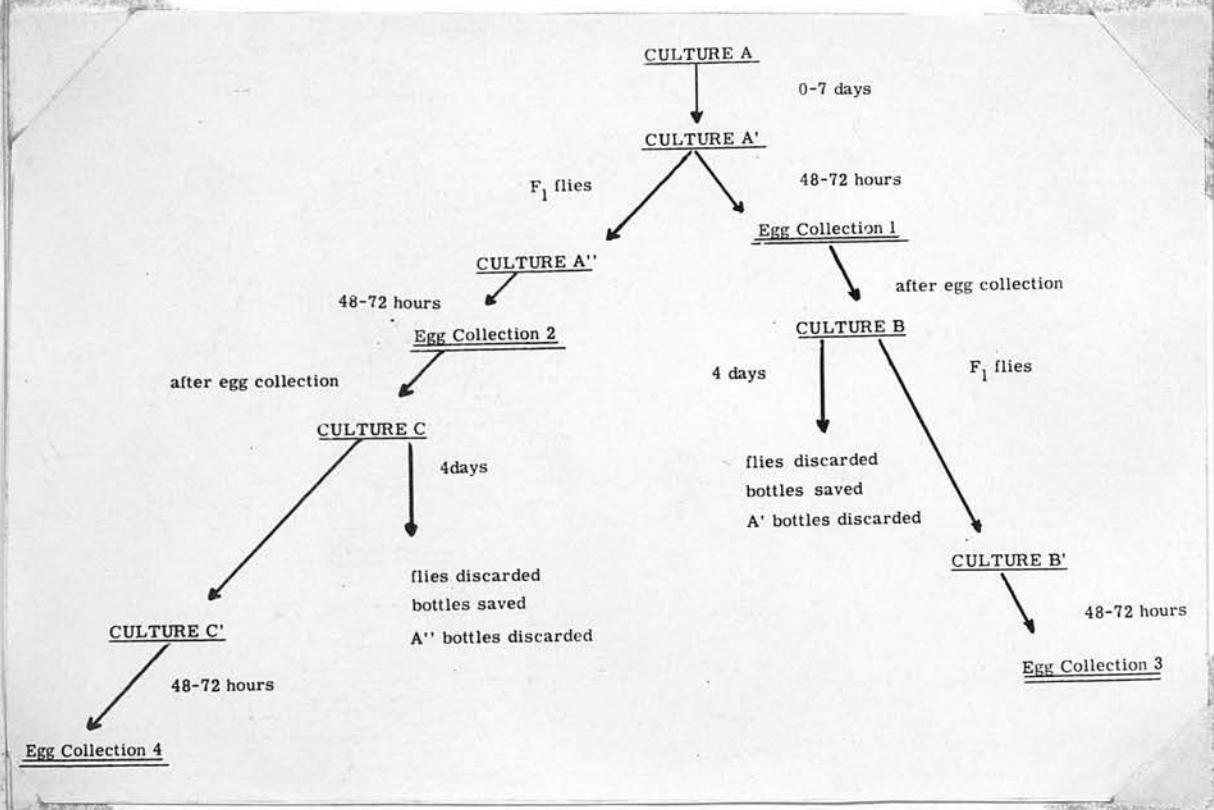
molasses-corn meal-agar medium seeded with a small quantity of Baker's yeast. These cultures were maintained at 25±1°C. Because of the desirability of collecting many thousands of eggs at one time from a particular stock, ordinary egg collection methods proved insufficient. It was found that old, poorly fed females gave inadequate numbers of eggs and even well-fed, young females gave poor egg counts when they had developed as larvae under poor nutritional conditions (i.e., crowding). Accordingly, before nutritional experiments were to be run, the stock of flies to be used were mass mated in ten half-pint milk bottles (designated Culture A); each bottle containing approximately 200-300 flies. Once larval development was observed in these bottles, the parent flies were discarded. The first filial generation flies, 0-7 days old, were shaken into ten freshly yeasted bottles (Culture A') and allowed to feed and lay eggs for 48-72 hours. At the end of this period, the flies were shaken into egg collection bottles (see description below) for the first egg collection. At this point Culture A' bottles were saved to await the eclosure of the F_2 generation flies. Following egg collection 1, the F_1 flies were shaken into ten freshly yeasted bottles (Culture B). After four days in the culture, these flies were discarded. Flies hatching in Culture A' were shaken over into fresh bottles of food (Culture A'') for two days to allow the females a sufficient feeding period before the second egg collection

which occurred approximately one week following the first collection. After egg collection 2, the flies were transferred to ten fresh bottles (Culture C) and allowed to remain there for four days before they were discarded. This general procedure gave two cultures of ten bottles each, staggered in such a way that egg collections could be made each week from young, well-fed, adult flies (see Figure 4 for a diagrammatic representation of stock maintenance).

The procedures used for the collection of eggs, their sterilization, and the subsequent inoculation of axenic larvae which are described below, were developed by Dr. J. H. Sang at the University of Edinburgh. Some minor modifications of these techniques as employed by Sang have been made. The procedures used are described in some detail since no full description of them have appeared in the literature.

Egg Collection: After the flies had been nourished on fresh yeast for two or three days, they were shaken into clean and dry empty one-quarter pint milk bottles capped by watch glasses containing an apple juice-agar mixture. Egg collection medium was prepared as follows: 97 ml of apple juice was measured out and placed in a 250 ml beaker. After bringing the juice to a boil on a hot plate, the beaker was removed and 3 gm of agar slowly added with constant stirring. 1 ml of glacial acetic acid and 2 ml of absolute ethyl alcohol were pipetted into the mixture. The suspension was again brought

Figure 4



Scheme Used to Insure Young, Well-fed Flies for the Collection of Large Numbers of Eggs.

to a boil to melt the agar. After a short cooling period, the medium was poured into watch glasses full to the brim. These were allowed to cool further and to solidify; once hardened, they were stored in the cold until ready for use. 100 ml of this medium was found sufficient for the preparation of twelve watch glasses.

Immediately before the flies were shaken into the egg collection bottles, the smooth hard surface of the apple juice-agar medium was sliced off and the resultant new surface roughened by scraping with a scalpel. In this way a more favorable oviposition site was presented to the females. It was observed that the flies would lay more eggs in a shorter space of time if a daub of live yeast, mixed with water to a thick paste, was added to the roughened surface. Usually, however, the yeast grew at too fast a rate on this medium so that difficulty was encountered in removing the eggs and sterilizing them. Subsequently, no yeast was added to the lids. It was discovered that a drop of water placed on the surface had the same beneficial effect as the live yeast; i.e., considerably larger numbers of eggs were deposited in a shorter time (see Spencer 1950, pp. 583-584). The watch glasses were secured to the bottles with strips of soft modeling clay (plasticene) which served both to secure the lids in place and prevent flies from escaping, and to act as legs when the bottles were inverted and placed in the incubator (see

Alpatov 1932; also see Robertson and Sang 1944).

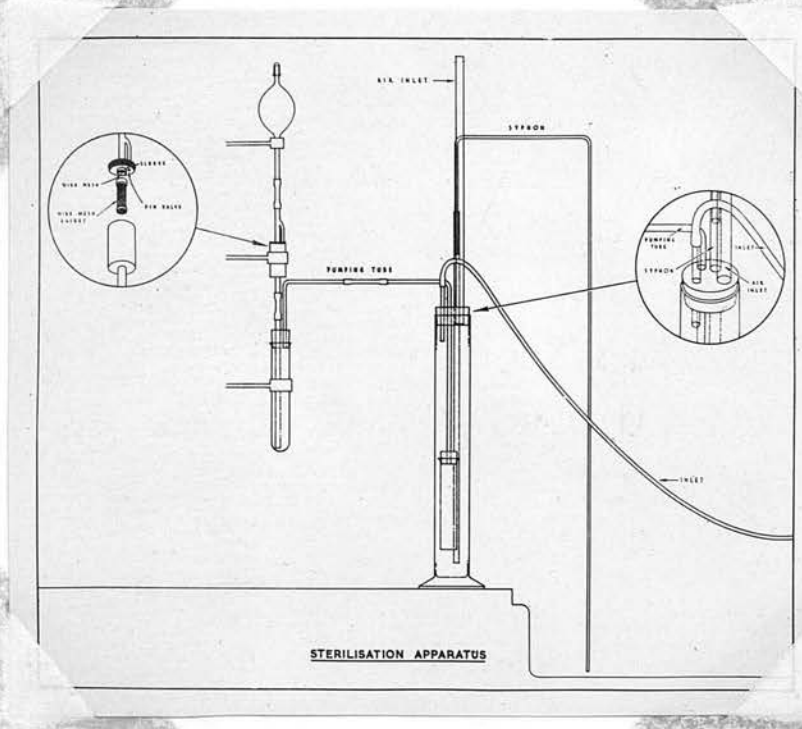
Cleansing and Sterilization of Eggs: Considerable difficulty was encountered periodically at the beginning of this investigation when attempts were made to obtain axenic cultures utilizing several different methods for washing and sterilizing eggs. The ornamental hexagonal and pentagonal figures on the outer surface of the chorion of the egg provide a rough surface for a tenacious attachment of yeast and bacterial spores. From our experience, it appeared that some of these spores were still present on the egg surface after the eggs had been washed and supposedly rendered aseptic. Therefore, the following procedure was inaugurated which has permitted the establishment of numerous experiments under axenic conditions with almost 100% success over a period of several years.

Sufficient numbers of eggs were usually collected on the apple juice medium within a 4 to 8 hour period after the flies were introduced into the collecting bottles. The newly laid eggs were brushed from the surfaces of the egg collection lids into a beaker containing insect Ringer's solution. Some fragments of medium were unavoidably brushed off in the process. These were separated from the eggs by pouring the contents of the beaker through a course wire sieve which retained the larger fragments but allowed the eggs to pass through. The resulting filtrate was poured through a finer mesh sieve which

retained the eggs but not smaller pieces of extraneous material. By means of a wash bottle containing distilled water, the eggs were further rinsed while still in the wire mesh sieve. They were removed from the sieve by tilting it slightly and washing the eggs into a clean baker with distilled water. A small amount of liquid detergent ("Glim") was found helpful in preventing the eggs from sticking to the sides of the beaker. A transference of the eggs was next made from the beaker to a previously sterilized wire mesh basket which, in turn, was placed immediately in a previously autoclaved egg sterilization apparatus (see Figure 5).

The sterilization apparatus, as pictured in Fig. 5, has been modified somewhat since the diagram was drawn, but the general mode of its operation has remained the same. Water, coming in through an inlet tube, filled the graduated cylinder and forced air over the "pumping tube" (containing sterile cotton) into a large boiling tube containing washing or sterilizing fluid (see below). This fluid was forced upward through a metal cylinder containing the egg basket, continuing upward to a glass bulb at the top of the apparatus. The lid of the metal cylinder contained a metal sleeve onto which the wire mesh basket was tightly fitted (see insert on left, Fig. 5). The center portion of the sleeve was covered with fine wire mesh to prevent the eggs from escaping into the fluid as it flowed upwards towards the glass bulb at the top. A pin

Figure 5



Egg Sterilization Apparatus

Drawn by Mr. E. D. Roberts, Institute of Animal Genetics, University of Edinburgh

(see text for an explanation of its operation)

valve was also located in the top of the metal cylinder to facilitate the flow of fluid around the basket as the liquid descended into the boiling tube at the bottom. A siphon was attached to the graduated cylinder which permitted continual operation of the apparatus since the tap water filling the cylinder siphoned off, thereby releasing the air pressure in the sterilization apparatus proper, causing the fluid to descend. In a short time (about 30 seconds), the whole washing and sterilizing cycle began over again.

After a number of trials, using several different sterilizing solutions, the following were adopted for use in the sequence given and for the times indicated:

1. Sterile insect Ringer's solution for 15 minutes.
2. Sterile 0.1% mercuric chloride solution for 15 minutes.
3. Filtered 70% ethyl alcohol for 30 minutes.

A variation of the technique described above involved the removal of the chorion from the egg by treatment for 30 minutes in approximately 0.1% solution of calcium hypochlorite (commercial "Clorox"). When this was done, the eggs were washed in the sterilization apparatus with distilled water followed by 40 minute exposures each to 1% "Cetavlon" (a synthetic quaternary ammonium compound) and 0.1% mercuric chloride solution. Because alcohol permeated the vitelline membrane of the egg, this fluid could not be used once the chorion was removed.

During the latter portion of the egg sterilization procedure the interior of an inoculation box (see Figure 6) was rinsed with calcium hypochlorite solution. An enclosed Hanovia germicidal lamp was turned on 15 minutes before the box was used. Following the alcohol wash, the entire metal cylinder containing the egg basket was removed from the sterilization apparatus and placed inside the inoculation box. The germicidal lamp was switched off at this time.

The inoculation box was an air-tight wooden box enclosing an ultra-violet light source for sterilization, a binocular microscope and a visible light source for the microscope. Two arm holes in the front fitted with elastisized denim sleeves permitted movement of hands and forearms within the box but prohibited the entrance of airborne contamination. Infection from the investigator was minimized by thorough hand and forearm washing before inserting them into the box. Once there, the tips of the fingers were dipped periodically into small beakers containing dilute calcium hypochlorite.

The eggs were removed from the basket by use of a sterile paper "spoon". This "spoon" was a modification of that figured by Begg and Sang (1950). It consisted of a rolled piece of stiff bond paper partially inserted into one end of a glass tube. A glass rod plunger, wrapped in a thin layer of cotton, had been previously inserted into the tube from the opposite end. The plunger served as a convenient means

Figure 6

Inoculation Box.

for ejecting the paper spoon when desired. In later experiments, cellophane dialyzing tubing was used in place of the bond paper for the manufacture of spoons. As mentioned in more detail below, this change offered several distinct advantages over the older technique.

The eggs were spread in a single layer on sterile agar in petri plates to prevent clumping and thereby facilitate maximum hatchability. The plates were incubated at 25-1°C until sufficient larvae were hatched for inoculation.

Inoculation of Larvae: The plates were returned to the inoculation box and the larvae picked up on the back of the paper spoon. Considerable care was taken not to crush the larvae when picking them up since it was discovered that even moderately rough handling was sufficient to kill them. The spoons with the adhering clump of larvae were carefully injected into the tubes of experimental medium by pressing on the glass rod plunger.

The cellophane dialyzing tubing made up into spoons greatly facilitated larval inoculations as the larvae adhered better to the cellophane than to the bond paper. The subsequent examination of tubes for the presence of pupae was made easier by the transparency of the cellophane on which a large number of larvae pupated, usually inside the rolled spoon.

Experiments performed in Edinburgh were done under somewhat different conditions than here described. A room, fitted

with a germicidal lamp in the ceiling, was used for inoculation purposes, obviating the necessity for the inoculation box previously described. All experiments done in Edinburgh utilized large boiling tubes containing 5 ml of medium per tube, whereas those performed in Amherst utilized 15 x 150 mm test tubes containing 4 ml of medium hardened on a slant. As the work developed, minor changes in technique were adopted insofar as they permitted easier handling of the material. Egg sterilization procedures were changed as new methods were devised, tested and found more efficient by Sang. During the course of the earlier experiments at Edinburgh, when success in establishing axenic cultures was quite erratic, the following washing and sterilization fluids were used: 2% Chloramine-T solution, White's fluid (consisting of 0.05% $HgCl_2$, 6.5% NaCl, 1.25% conc. HCl and 50% ethyl alcohol), 2% sodium benzoate, 5% antiformin in 1% "Teepol" (a liquid detergent) solution and 75% glycerin. These fluids were eventually replaced by those previously described.

Measurement of Growth and Development: Attempts were made, throughout the establishment of each axenic culture (especially later ones), to standardize the experimental procedures just described. This was done in an effort to achieve homogeneity of results (as expressed in terms of numbers of pupae and adults formed, and the time taken by the larvae in prepupal life) within each experimental set containing tubes

with a germicidal lamp in the ceiling, was used for inoculation purposes, obviating the necessity for the inoculation box previously described. All experiments done in Edinburgh utilized large boiling tubes containing 5 ml of medium per tube, whereas those performed in Amherst utilized 15 x 150 mm test tubes containing 4 ml of medium hardened on a slant. As the work developed, minor changes in technique were adopted insofar as they permitted easier handling of the material. Egg sterilization procedures were changed as new methods were devised, tested and found more efficient by Sang. During the course of the earlier experiments at Edinburgh, when success in establishing axenic cultures was quite erratic, the following washing and sterilization fluids were used: 2% Chloramine-T solution, White's fluid (consisting of 0.05% $HgCl_2$, 6.5% NaCl, 1.25% conc. HCl and 50% ethyl alcohol), 2% sodium benzoate, 5% antiformin in 1% "Teepol" (a liquid detergent) solution and 75% glycerin. These fluids were eventually replaced by those previously described.

Measurement of Growth and Development: Attempts were made, throughout the establishment of each axenic culture (especially later ones), to standardize the experimental procedures just described. This was done in an effort to achieve homogeneity of results (as expressed in terms of numbers of pupae and adults formed, and the time taken by the larvae in prepupal life) within each experimental set containing tubes

with identical media. It was observed that time spent in the pupal stage was relatively unaffected under the experimental conditions used. Nutritionally sub-optimal media, rough handling of larvae during inoculations, sub-optimal larval inoculum per tube, and poor medium consistency (too hard or too soft a gel), however, all tended to reduce the numbers of surviving larvae within the tubes. Whenever these factors, or any combination of them, were operative in some tubes but not others, within the same experimental set, it was obvious that heterogeneous results would ensue. Care was taken, therefore, to ensure that these factors played as small a role in this investigation as possible. Media consistencies, made by a concentration of 15 mg/ml of agar for the amino acid media and 25 mg/ml of agar for the casein media, were found to be optimal for the developing larvae. Experiments were run to test the optimal number of larvae to be inoculated into the 15 x 150 mm test tubes and found to be 30 (Ellis, unpublished). Sang (personal communication) has found 40 larvae per tube to be optimal when boiling tubes are used. In some of our experiments only 25 larvae per boiling tube were inoculated, although this number was increased in later experiments.

As stated earlier, the natural medium for Drosophila contains yeast. As our experiments were concerned with a nutritional requirement for nucleic acid, this organism, even

though killed, could not be used. In the absence of a chemically defined medium which permits optimal larval growth and development, all our experiments were necessarily performed using sub-optimal media for larval development. The use of these media increased the length of larval life and decreased larval survival. The distribution curves of pupation and adult eclosion times exhibited a pronounced "skewness", with the "tail" of the curve extended to the right as a function of increased larval life. There is evidence that a portion of this "skewness" is inherent in Drosophila (Sang, personal communication) but a considerable amount may be attributable to the factors mentioned above; i.e., lengthened larval life and decreased larval survival on sub-optimal media.

It was found imperative, therefore, to avoid final calculations which incorporated results from tubes whose larval and adult frequencies showed marked heterogeneity with respect to the remaining tubes within the experimental set (identical medium), and whose pupal and eclosure time distributions were heterogeneous. Tubes within an experimental set showing obvious and marked heterogeneity, by inspection of the data, were excluded from the final counts. We do not feel that the elimination of these tubes in any way biases our results; rather, their inclusion in the final tabulations would impart such a bias.

Of the two major types of media used, that containing

casein gave much less heterogeneous results than that containing the amino acids. Media containing amino acids were used in the early experiments, however, because it was not known at that time whether or not significant amounts of nucleic acid (or its constituent purines and pyrimidines) would be present as contamination in successive batches of casein to be used. Through the courtesy of Dr. V. C. Dewey of Amherst College, both British and American casein samples have now been assayed for purine and pyrimidine activity. Tetrahymena pyriformis W was used as test organism. She found no appreciable activity for either of the two nucleic acid component moieties in either sample of casein.

The tubes were examined each day until pupation began. Except where noted, each tube was then examined once every 24 hours at approximately the same time during each day. The number of pupae formed during these periods was recorded together with the time taken by the larvae to reach this stage. When eclosure occurred, the number and time for the emerging adults during each 24 hour period was also recorded. At the termination of an experiment, the adult flies were shaken from the tubes, etherized, and recounted. This procedure served as a check on the accuracy of counts made by inspection of the living flies inside the tubes. Counts were also made at this time of the empty pupal cases, as well as any which still contained pupae that had failed to develop

further.

The data are presented in the text as follows:

1. The total initial larval inocula in an experimental set (column headed "initial larvae").
2. The percentage of larvae inoculated that pupated ("% pupation").
3. The median time from inoculation of the larvae to pupation expressed in days ("median pupation time").
4. The percentage of adults eclosing from the pupae formed ("% eclosion").
5. The median time from inoculation of the larvae to eclosion expressed in days ("median eclosion time").
6. The column headed "reference" refers to the data collected for each experimental tube. These individual tube data are appended to the thesis as Appendix C and include tubes which showed obvious heterogeneity. These latter tubes are not included in the final tabulations, however.

Median pupation and eclosion times were calculated in place of mean times (at the suggestion of Sang) as descriptive indices of pupation and emergence distributions. Because of the typical "skewness" of the distributional curves already mentioned, statistical analyses of the data cannot be properly made until scaler transformations that will "normalize" the distributions are available. The median time values, which

measure the mid-points of the distributions regardless of the "skewness" involved, will then be little affected by such scaler transformations.

Spot checks were occasionally made for infections of some of the tubes in each experiment. This was done by pipetting 5 ml of sterile nutrient broth into each tube. These were then incubated at 37°C for 48 hours. Only rarely were infections, not previously seen by visual inspection of the tubes, determined in this manner. Results obtained from any tubes showing the presence of microorganisms were eliminated from the final tabulations.

EXPERIMENTAL

Experiments utilizing British and American Amino Acids: Having succeeded in culturing Drosophila melanogaster larvae on a synthetic diet containing free amino acids, our first experiment at Edinburgh was designed to duplicate that success. Accordingly, Medium 3 (Table 1) was made up as previously described. In addition, varying amounts of ribonucleic acid (RNA) were added to a series of equal portions of the base medium since a dose response to this substance was desired. One portion of the medium contained no nucleic acid. Eggs were obtained from females of Oregon-K (Ore-K), a stock reported by Sang (personal communication) to require RNA as mentioned previously. The eggs were washed and rendered axenic by the procedures outlined in Begg and Sang (1950). The newly-emerged larvae were inoculated onto the sterile synthetic medium. By fifteen days following inoculation all larvae were dead, a majority of them failing to reach the 2nd instar of larval development.

This result prompted research along three main lines. First, it was felt that Oregon-K might require in its diet an unknown factor or factors not required by other stocks that had been successfully reared on similar synthetic media. This possibility was soon eliminated by our failure to achieve larval growth and development with any of nine stocks tested

on the medium. The stocks tested were: Ore-K (retest); Ore-S; Ore-R; Samarkand; wild Edinburgh (WE_4); Nettlebed-inbred (Nb_9); Nettlebed-large (I,20); Nettlebed-long thorax (2LT) and In (2LR)40d/Cy sp^2. The last stock was one which had previously been raised successfully on a duplicate of the synthetic medium now under consideration. Its failure to grow at this time proved conclusively that the medium as now constituted, rather than the stock of Drosophila used, was responsible for the early larval deaths observed.

A second possibility for our failure to achieve larval growth and development was believed to be due to an unnoticed change in procedural technique in the preparation of the medium. Consequently, media were prepared in a variety of ways (i.e., the sequence of additions to the medium of its components was altered; the time taken for autoclaving the media was reduced; pasteurization procedures were employed in place of autoclaving as a means of media sterilization, etc.) after the concentrations of all components of the diet were rechecked and the stock solutions made up anew. None of these changes had any effect in permitting larval development beyond the 2nd instar.

There remained a third possibility. This was that a substance (or substances), toxic to Drosophila larvae growing on a purified diet, was being incorporated into the medium as a contaminant of the chemicals used. With a synthetic medium

as complex as that used for Drosophila, the determination of which component of the diet was responsible for the adverse effect on larval development presented a problem difficult to overcome.

A series of experiments were set up which consisted of amino acids obtained from different commercial firms (British Drug Houses, Ltd. (BDH); L. Light and Co., Ltd. (Light's); and F. Hoffmann-LaRoche and Co., Ltd. (Roche)) wherever possible. No substitution of an amino acid from one firm by that from another was found effective in permitting larval development. Similar experiments were run in which the remaining components of the diet (i.e., salts, sterol, vitamins and RNA) were obtained from different commercial sources. The RNA was purified according to the procedures of Fletcher, Gulland, Jordan and Dibben (1944). Varying concentrations of these components were also utilized. No pupae appeared in any of these experiments; most larvae failed to reach even the 2nd instar stage. These entirely negative results were interpreted as indicating that the amino acid component of the diet was at fault rather than the salts, sterol, vitamins or nucleic acid.

Amino acids from the Nutritional Biochemicals Corporation (NBC), Cleveland, Ohio, USA had previously been used successfully in our earlier Amherst experiments (see Appendices A and B). Upon receipt of these at Edinburgh, comparisons of these

acids with those from British sources were made using the paper chromatograph technique. (We wish to thank Dr. G. W. Kidder of the Biological Laboratories, Amherst College, for sending us the NBC amino acids, and Dr. A. Kemble of the Department of Chemistry, University of Edinburgh, for performing the chromatographic experiments for us.) No appreciable differences in the locations of the amino acids from British and American sources after their movement in the solvent on the paper strips could be found.

A further experiment was set up incorporating 13 NBC amino acids in a medium otherwise containing wholly British-produced chemicals. In addition, the experiment was designed so that each NBC amino acid in turn was replaced by a comparable amino acid from a British source in a further set of experimental tubes. In a final set, a repetition of the medium which contained all 13 amino acids from British sources was also set up. The results from this experiment are presented in Table 3. It can be seen from the data presented that some larval growth and development occurred within each experimental set (all tubes in the tryptophan series became infected and were discarded) with the exception of larvae raised on the medium containing all its amino acids from British sources. From these results it was apparent that whatever toxic substances were present as contamination of the British amino acids, were not present in sufficient quantity

TABLE 3

RESULTS OF EXPERIMENT IN WHICH EACH NBC AMINO ACID WAS REPLACED BY A SINGLE BDH, ROCHE OR LIGHT'S AMINO ACID.

(This experiment began with 90 tubes, 70% of which became infected and were discarded)

Medium	Initial Larvae	% Pupation	Mean Pupation Time	% Eclosion
			days	
13 NBC Amino Acids	25	52.0	10.0	84.6
12 NBC A.A. + BDH arginine	50	34.0	16.6	35.3
" + BDH cystine	75	41.3	16.9	35.5
" + BDH glutamic	25	28.0	17.0	85.7
" + BDH glycine	25	80.0	10.0	100.0
" + BDH histidine	50	66.0	13.3	84.8
" + BDH isoleucine	50	30.0	17.0	86.7
" + Roche leucine	75	16.0	14.6	66.7
" + BDH lysine	75	44.0	14.4	69.7
" + Light's methionine	25	4.0	11.0	100.0
" + Roche phenylalanine	50	46.0	15.1	69.6
" + Roche threonine	25	16.0	14.5	100.0
" + BDH tryptophan	-	-	-	-
" + BDH valine	50	36.0	16.7	44.4
13 BDH etc. Amino Acids	75	0.0	-	-

in any single acid (with the possible exception of tryptophan) to prevent larval development.

Experiments with Oregon-K utilizing whole casein media: Our failure to achieve larval development on a synthetic medium, in which all components were defined chemically, obligated our undertaking experiments which utilized media containing whole casein in place of the amino acids as nitrogen source. It has already been pointed out that the casein used in these experiments contained no appreciable purine or pyrimidine activity for the protozoan, Tetrahymena pyriformis W, which was used as assay organism for the detection of these compounds in casein samples. We may conclude, therefore, that growth and development occurring on RNA-deficient media is attributable wholly to the synthetic capacities of the larvae raised on the casein media.

A medium containing casein (Medium 1, Table 2) was prepared and divided into two equal portions. To one portion, 3 mg/ml RNA was added, while the remaining half received no addition of nucleic acid or its constituents. Oregon-K, reported as inbred for 731 generations at that time (see Sang and McDonald 1954), was used to test its ability to develop on a diet lacking RNA. A similar experiment was run which tested the synthetic capacity of Edinburgh-large (EL_2) in the same way. The results of these experiments are presented in Table 4.

TABLE 4

Medium 1, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
3 mg/ml RNA	Ore-K	150	72.7	10.1	99.1	14.0	169-171
0 mg/ml RNA	"	200	17.3	23.1	34.6	26.2	286-289
1 mg/ml RNA	EL_2	125	63.2	11.0	98.7	16.1	177-181
0 mg/ml RNA	"	100	58.0	12.8	86.3	17.7	224-228

As can be seen from the data presented, Oregon-K showed a marked reduction in the numbers of pupae and adults produced, on a medium lacking nucleic acid, over that shown on a medium containing RNA. In addition, the time taken to reach these stages of the life cycle was considerably increased on RNA-deficient media over that shown on RNA medium. Although some stimulation of growth and development occurred with EL_2 larvae on medium containing RNA, no great reduction in numbers of pupae and adults formed, nor increase in the time taken to reach these stages, took place when the larvae received no exogenous RNA. These results indicate that the synthetic capacity for RNA production in EL_2 is much greater than that of Oregon-K. Thus, we have considered Oregon-K to require RNA in its diet for growth and development of the larvae. It should be noted, however, that the requirement for RNA is not absolute; 17.3% of the original larval inoculum pupated and 34.6% of these were able to complete metamorphosis and eclose as adult flies on medium containing no RNA. It would, perhaps, be more suitable to designate this stock as "RNA-stimulated" rather than as "RNA-requiring" were it not for the fact that all Drosophila stocks tested to date are stimulated to some degree by the presence of RNA in their diets. In Oregon-K, as well as in In (2LR)40d/Cy sp^2, this stimulation is considerably greater than in other stocks tested.

To test the possibility that the few Oregon-K adults obtained on RNA-deficient medium were products of a genetically heterogeneous population in which a small minority were capable of synthesizing their own RNA while the majority could not do so, all adults appearing on a RNA-deficient medium were shaken over to food vials and their offspring raised on ordinary laboratory culture medium. After several generations to allow time for an increase in the size of the selected population, axenic larvae were inoculated onto a medium similar to the one just described (i.e., "plus" and "minus" RNA). The results of this experiment are given in Table 5. They indicate that this stock is neither genetically heterogeneous with respect to the factor or factors responsible for the anomaly of RNA synthesis found, nor had genetic reversion to a nutritional RNA wild-type occurred in those larvae that reached the adult stage on RNA-deficient media.

Further experiments were run in attempts to determine which component of RNA was the limiting factor in RNA synthesis for this stock. Medium 1 (Table 2) was prepared which lacked RNA but to which the purine and pyrimidine bases (except cytosine, which was unavailable at the time) and their ribosides and ribotides were added singly to separate experimental sets. Numerous tubes within the experiment became infected necessitating the removal of most of the sets from the experiment. The results we did obtain from uninfected tubes

TABLE 5

Medium 1, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
2.5 mg/ml RNA	Ore-K select.	200	63.0	8.9	99.2	13.1	95-98
0.0 mg/ml RNA	"	250	5.6	17.0	64.3	23.8	281-285

TABLE 6

Medium 1, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
0.11 mg/ml adenine	Ore-K	200	17.5	12.7	80.0	16.2	229-232
0.11 mg/ml guanine	"	150	29.3	10.6	70.5	14.4	182-184
0.11 mg/ml uracil	"	200	-	-	-	-	-
0.165 mg/ml cytidine	"	150	56.0	11.4	94.0	15.0	185-187

are presented in Table 6.

These data, although based on a small number of experimental tubes, show that the presence of the purine bases, adenine and guanine, in the medium markedly affected the developmental rate of the larvae but failed to increase significantly the yield of pupae formed (cf. 17.3% pupation obtained with RNA-deficient medium with 17.5% and 29.3% pupation obtained when adenine or guanine was supplied respectively). When the pyrimidine riboside, cytidine, was present in the medium, on the other hand, both the rate of larval development and the numbers of pupae and adults formed were markedly affected (cf. 56% pupation, 94% eclosion from pupae in 15 days with only 17.3% pupation and 34.6% eclosion in 26.2 days). We conclude from this information that the synthesis of cytidine is limited in Oregon-K. No pupation occurred on the medium containing the pyrimidine base, uracil. One might expect uracil to be converted to its riboside, uridine, which in turn could be aminated to form cytidine. This latter reaction is known to occur readily in many organisms. The complete absence of pupae in the uracil series suggests that some uncontrolled factor was responsible for the lack of larval development rather than an inability on the part of the organism to perform one of the reactions mentioned above, although both possibilities might be present. Data obtained in earlier experiments (Ellis 1950) showed that larval growth and de-

velopment of Oregon-R and In (2LR)40d/Cy sp^2 were depressed whenever free uracil was present in the medium, suggesting that this compound, in the free state, is toxic to Drosophila larvae grown on a purified diet. We are unable, therefore, to draw any conclusion concerning the metabolic role of pyrimidine compounds in RNA synthesis in Oregon-K, based on these data.

Experiments with Stocks 1, 2 and 3 on casein media: Based on the assumption that the nucleic acid requirement of Oregon-K was primarily due to a single mutant gene, three stocks of Drosophila which contained homozygous Oregon-K chromosomes within a background of chromosomes derived from EL_2 were made up as previously described (see Figure 3). It was believed that the gene within a chromosome responsible for the inability of Oregon-K to synthesize its own RNA would be revealed by rearing the larvae from each of these three stocks on RNA-deficient media. For example, if the gene was located on the second chromosome, and was either dominant or recessive, we would expect Stock 2, containing its second chromosomes derived from Oregon-K and its X and 3rd chromosomes from EL_2 - a stock known to be capable of synthesizing its own RNA - to produce only a few pupae and adults in a greatly increased length of time as compared to that obtained when RNA was added to the medium. The remaining stocks, lacking the 2nd chromosome factor, would be expected to grow and

develop on RNA-deficient media at least as well as EL_2 alone. In addition to nutritional tests on RNA-deficient media, it was felt that dose responses to varying concentrations of RNA might prove useful in substantiating evidence obtained from the RNA-deficiency experiments. A decrease in the optimal amount of RNA for larval growth and development, supplied exogenously, was expected to be found for those stocks which no longer required RNA. Furthermore, the RNA-requiring stock was expected to show a rather sharp response to a particular concentration of RNA.

A preliminary experiment, consisting of casein base medium (Medium 1, Table 2) with and without a RNA supplement, was set up. The data obtained are presented in Table 7. This experiment proved unsuccessful for a number of reasons. Only a small number of larvae could be inoculated per tube due to a small initial egg collection. For the same reason, only a small number of tubes in each series were inoculated, and of these several showed infections with microorganisms beginning at about the 12th day following inoculation.

A considerable improvement in the base medium was achieved by Sang at this time, and the new diet was utilized in subsequent experiments. Axenic larvae from Stocks 1, 2 and 3 were inoculated onto Medium 2 (Table 2) which had been prepared with varying concentrations of RNA in separate experimental series. The data obtained from these experiments are

TABLE 7

Medium 1, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
8 mg/ml RNA	1	125	-	-	-	-	-
0 mg/ml RNA	1	125	10.4	10.4	92.3	14.2	172-176
8 mg/ml RNA	2	20	50.0	7.7	100.0	14.0	88-89
0 mg/ml RNA	2	125	0.0	-	-	-	-
8 mg/ml RNA	3	40	65.0	9.1	100.0	12.7	90-94
0 mg/ml RNA	3	50	4.0	13.5	0.0	-	-

TABLE 7

Medium 1, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
8 mg/ml RNA	1	125	-	-	-	-	-
0 mg/ml RNA	1	125	10.4	10.4	92.3	14.2	172-176
8 mg/ml RNA	2	20	50.0	7.7	100.0	14.0	88-89
0 mg/ml RNA	2	125	0.0	-	-	-	-
8 mg/ml RNA	3	40	65.0	9.1	100.0	12.7	90-94
0 mg/ml RNA	3	50	4.0	13.5	0.0	-	-

presented in Tables 8, 9 and 10.

From an examination of the results obtained in this experiment, it is clear that the expectation concerning low numbers of pupae and adults confined to a single one of the three stocks tested on RNA-deficient medium is far from realized. The percentages of pupation on nucleic acid-less media, 15.6% for Stock 1, 22.0% for Stock 2 and 19.3% for Stock 3, all represent markedly reduced numbers of pupae over those formed in the presence of RNA. Moreover, considering the best median eclosion times which measure the over-all performance of the various stocks on the media better than any other single figure, there appears to be no sharp delimitation of better growth at an optimal concentration of exogenously supplied RNA in any of the stocks. In all three stocks, the optimal concentration of RNA appears to be between 2 and 4 mg/ml. There are a number of possible explanations for these anomalous results, and discussion of them will be reserved for a later section of this thesis.

Experiments with Stocks 4, 5 and 6 on casein media: A further attempt was made to localize on the chromosomes the factor responsible for the inability of Oregon-K to synthesize enough RNA for its metabolic requirements during larval growth and development. This was done utilizing a further series of three stocks (designated Stocks 4, 5 and 6) which were kindly supplied to us by Mr. B. K. Sen of the Institute of Animal

TABLE 8

Medium 2, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
8 mg/ml RNA	1	200	52.2	6.6	75.2	11.4	20-23
6 " "	"	200	70.0	7.8	88.6	11.7	24-27
4 " "	"	200	68.0	7.4	80.9	11.4	28-31
3 " "	"	200	73.0	6.8	87.0	11.5	32-35
2 " "	"	150	53.3	8.1	87.5	12.5	36-38
1.5 " "	"	150	80.0	8.3	79.2	12.4	39-41
1 " "	"	200	65.5	9.6	86.3	13.5	152-155
0 " "	"	250	15.6	10.6	53.8	14.4	156-160

TABLE 9

Medium 2, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
8 mg/ml RNA	2	100	48.0	8.0	97.9	11.8	127-130
6 " "	"	150	31.3	9.1	97.9	13.5	161-164
4 " "	"	150	52.7	8.0	100.0	11.9	131-133
3 " "	"	150	60.7	8.2	100.0	11.7	141-143
2 " "	"	150	50.7	8.2	97.4	11.5	134-136
1.5 " "	"	200	66.5	8.0	99.2	11.7	144-147
1 " "	"	150	48.7	8.6	98.6	12.7	137-140
0 " "	"	200	22.0	9.8	68.2	13.6	148-151

TABLE 10

Medium 2, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
8 mg/ml RNA	3	200	64.0	8.7	96.9	13.0	42-45
6 " "	"	200	50.5	8.4	90.1	11.8	46-49
4 " "	"	150	65.3	8.3	100.0	11.2	50-53
3 " "	"	150	61.3	8.6	93.5	12.3	54-56
2 " "	"	150	45.3	8.6	98.5	12.2	57-59
1.5 " "	"	150	65.3	9.4	93.9	13.1	124-126
1 " "	"	100	51.0	10.1	90.2	13.7	165-166
0 " "	"	150	19.3	10.6	58.6	14.8	204-206

Genetics, University of Edinburgh. These stocks had been made up by Sen in a similar manner to that described for Stocks 1, 2 and 3. Wild-Edinburgh (WE_3) Drosophila, however, had been used in place of EL_2 in these crosses. Stocks 4, 5 and 6 respectively, therefore, were constituted as follows: K/K;WE/WE;WE/WE - WE/WE;K/K;WE/WE - WE/WE;WE/WE;K/K.

WE_3 stock was tested separately to determine its ability or lack of ability to synthesize enough RNA for its metabolic needs during development. It was found that this stock, like EL_2, could synthesize its own RNA from the components of its diet. The data illustrating this are given in Table 11.

Similar results to those obtained with Stocks 1, 2 and 3 were found when Stocks 4, 5 and 6 were raised on casein media without nucleic acid added. The observed percentages of 22.0% for Stock 4, 15.0% for Stock 5 and 18.0% for Stock 6, were all much lower than the percentages found when the larvae were grown in the presence of RNA. The tabulated results obtained in these experiments are also presented in Table 11.

Experiments with Cy/Pm;D/Sb on amino acid media: At Amherst, a new approach to the problem of the chromosomal localization of the RNA requirement in Oregon-K was undertaken. The method chosen, ignoring for the time being the possibility that the sex chromosome or the 4th chromosome contained the mutant gene, consisted of crossing a stock whose 2nd and 3rd chromosomes were marked by dominant characters,

TABLE 11

Medium 2, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
2.5 mg/ml RNA	WE_3	150	63.3	8.1	97.9	11.6	60-62
0.0 mg/ml RNA	"	100	56.0	11.7	89.3	15.1	167-168
2.5 mg/ml RNA	4	50	46.0	8.9	100.0	12.8	63-64
0.0 mg/ml RNA	"	50	22.0	12.8	90.9	17.0	233-234
2.5 mg/ml RNA	5	200	47.5	8.2	96.8	11.9	65-68
0.0 mg/ml RNA	"	100	15.0	15.1	60.0	18.5	238-239
2.5 mg/ml RNA	6	200	43.5	8.0	98.8	12.4	69-72
0.0 mg/ml RNA	"	150	18.0	13.1	55.6	16.4	235-237

and in which crossover suppressors were also present, with Oregon-K. By rearing the resultant larvae on a synthetic nucleic acid-less medium under axenic conditions and analyzing the classes of adults that appeared, it was hoped that the requirement, if dominant, would be localized on one of the two major autosomes in this manner. If the requirement proved to be a recessive, subsequent crosses between the F_1 flies, together with further nutritional tests on nucleic acid-less media to produce an F_2, would reveal the presence of a recessive gene if it were present in either the 2nd or the 3rd chromosome.

The dominant marker stock selected which fulfilled the above specifications was Cy, sp^2/Pm, dp b; DcxF, ru h ca/In (3R)Mo, Sb. For simplification we have designated this stock as Cy (Curly)/Pm (Plum);D (Dichaete)/Sb (Stubble) which indicates only the dominant traits in its make-up.

Our first experiments with Cy/Pm;D/Sb consisted of a series of nutritional tests on Media 1, 2 and 3 (Table 1), with and without an added nucleic acid supplement, to determine its ability to synthesize its own RNA. The results found are given in Table 12.

To our surprise, this stock appeared to be a nucleic acid-requirer also. Only six pupae were formed out of 600 larvae originally inoculated onto the 3 different nucleic acid-less media used, and all six pupae failed to eclose. The

TABLE 12

Medium 1, Table 1 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
1 mg/ml RNA	Cy/Pm;D/Sb	100	18.0	11.2	88.9	14.4	215-218
0 mg/ml RNA	"	400	0.0	-	-	-	-
Medium 2, Table 1 +							
1 mg/ml RNA	Cy/Pm;D/Sb	150	20.0	14.5	23.3	17.3	267-272
0 mg/ml RNA	"	100	0.0	-	-	-	-
Medium 3, Table 1 +							
1 mg/ml RNA	Cy/Pm;D/Sb	125	17.6	11.8	81.8	15.2	210-214
0 mg/ml RNA	"	100	6.0	14.0	0.0	-	263-266
Laboratory Medium	Cy/Pm;D/Sb	300	31.7	3^{+}	84.2	7.4	1-10

percentages of pupae obtained from media containing 1 mg/ml RNA, however, were small in all 3 sets. This fact necessitated a further experiment using standard laboratory medium containing live yeasts to determine the viability of this mutant under ordinary laboratory conditions. The data from the experiment are included in Table 12. They indicate that only a small number (31.7%) of pupae would be expected to be formed even under the best nutritional conditions we are able to supply, and that the low pupal percentages obtained by use of synthetic media are not solely due to the inadequacy of the media to support growth and development.

The effect of pH on growth and development of Drosophila in casein and amino acid media: From consideration of the above results, it was obvious that Cy/Pm;D/Sb would not fulfill the requirement for a marker stock capable of synthesizing its nucleic acid from a diet lacking this substance. It was decided, however, to retain the general approach to the problem of the localization of RNA requirements within the chromosomal complement, but to attempt such a localization in Cy/Pm;D/Sb rather than in Oregon-K. This decision led to a series of crosses with a phenotypically wild-type stock. Oregon-R, known to be capable of synthesizing its own RNA (see Appendix B).

While these experiments were in progress, a control series was added which retested the requirement for exogenous

RNA in both Cy/Pm;D/Sb and Oregon-R. Because Cy/Pm;D/Sb larvae grew poorly even on a live yeast diet, a medium containing casein (Medium 3, Table 2; a medium found by Sang (personal communication) to improve growth and development) was prepared in order to obtain growth as similar as possible to that found when yeasts are incorporated into the diet.

Considerable difficulty was encountered at this time in determining the optimal concentration of agar to be used in this medium. An entire experiment was discarded because of too soft a gel of the medium. Upon re-preparation, the medium was not neutralized with 1N NaOH so that its hydrogen-ion concentration remained at about pH 5.0 rather than pH 7.0. Surprisingly, 35.8% of the axenic Cy/Pm;D/Sb larvae inoculated onto the nucleic acid-less medium with a low pH were able to pupate; a percentage pupation that actually exceeded that obtained when Cy/Pm;D/Sb larvae were inoculated onto media containing live yeasts.

The next experiments were designed to examine the apparent pH effect more quantitatively. Accordingly, the hydrogen-ion concentrations of equal portions of the complete medium (Medium 3, Table 2), with and without added nucleic acid, were varied by the addition of 1N NaOH or 1N HCl. Final pH readings of the media were made with a Beckman glass electrode pH meter. In (2LR)40d/Cy sp^2 larvae were inoculated onto these media since Oregon-K had been lost from our stocks at this

time and a known RNA-requirer was needed to test under these conditions. The summarized results from the experiment are given in Table 13.

They show that on a medium containing casein but lacking RNA the larvae were able to pupate in nearly as great a number as when nucleic acid is added to the medium, provided that the hydrogen-ion concentration of the medium is high. The same medium at pH 7.3 permitted only 22.5% of the original larval inoculum to pupate, a percentage within the range found by Hinton (1955) for this stock on a nucleic acid deficient amino acid medium. The rate of larval development, however, is much faster on the casein medium (cf. median pupation time on casein of 11.7 days with mean pupation time of 21.5 to 26.0 days on an amino acid medium). It was imperative, therefore, to test In (2LR)40d/Cy sp^2 on a medium containing amino acids but lacking RNA to determine whether or not the RNA requirement had possibly been lost before IN (2LR)40d/Cy sp^2 was tested on the casein medium.

The above experiment was run, and the data obtained are presented in Table 14. They give a clear indication that the requirement is not lost when the larvae are raised under these conditions. It should be noted, furthermore, that the hydrogen-ion concentration in the amino acid medium has little or no effect on larval development, at least within the range from pH 4.0 to pH 7.0.

TABLE 13

Medium 3, Table 2 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
4 mg/ml RNA, pH 5.4	In (2LR)40d	270	71.1	6.4	99.5	11.1	11-19
" pH 6.5	"	180	34.4	9.1	96.8	11.9	73-79
" pH 7.0	"	240	24.2	10.7	96.6	15.9	188-195
" pH 8.7	"	270	0.0	-	-	-	-
0 mg/ml RNA, pH 5.3	In (2LR)40d	240	67.1	10.1	88.8	14.5	110-117
" pH 6.6	"	180	32.8	8.7	84.7	13.1	118-123
" pH 7.3	"	240	22.5	11.7	92.6	15.6	196-203
" pH 8.3	"	240	0.4	13.0	100.0	17.0	255-262

TABLE 14

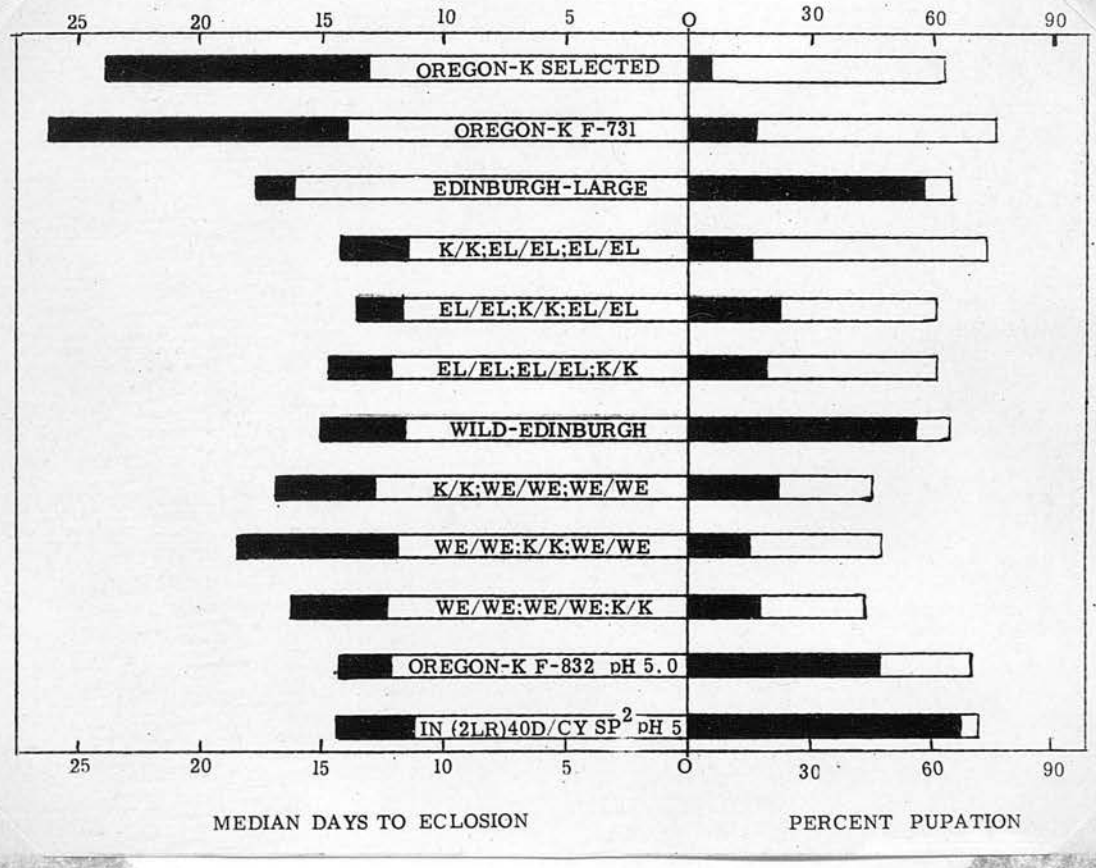
Medium 3, Table 1 +	Stock	Initial Larvae	% Pupation	Median Pupation Time	% Eclosion	Median Eclosion Time	Reference
				days		days	Tube no.
4 mg/ml RNA, pH 4.0	In (2LR)40d	240	40.0	16.1	84.4	19.6	273-280
" pH 5.0	"	240	40.4	14.0	91.8	18.2	240-247
" pH 6.0	"	180	44.4	14.2	91.2	17.8	248-254
" pH 7.0	"	150	58.0	13.2	92.0	17.3	219-223
0 mg/ml RNA, pH 4.0	In (2LR)40d	300	0.0	-	-	-	-
" pH 5.0	"	300	0.0	-	-	-	-
" pH 6.0	"	300	0.0	-	-	-	-
" pH 7.0	"	300	3.3	22.0	60.0	26+	-
" pH 8.0	"	300	0.0	-	-	-	-
Medium 3, Table 2 +							
4 mg/ml RNA, pH 5.0	Ore-K F-843	240	70.8	7.9	87.1	12.2	80-87
0 mg/ml RNA, pH 5.1	"	240	47.1	10.6	85.8	14.4	99-106

Recently, a new culture of Oregon-K was received at Amherst from Dr. J. H. Sang which has been inbred for 843 generations. Unfortunately, its viability and fecundity has been greatly reduced so that considerable difficulty was experienced in obtaining sufficient eggs to sterilize by the procedures previously described. Consequently, we were able to inoculate but a single experimental series (out of 5) in an experiment similar to that used with In (2LR)40d/Cy sp^2.

The series inoculated with axenic Oregon-K larvae contained Medium 3 (Table 2) with and without added RNA at pH 5.0 and 5.1 respectively. A marked increase in the number of pupae formed over that expected (see Table 14) is suggestive that Oregon-K reacts in a similar manner as In (2LR)40d/Cy sp^2 to high concentrations of hydrogen ions in media containing casein but lacking RNA.

Results from several of the experiments described above are presented graphically in Figure 7. Consideration of this Figure will be presented in the following section of this thesis.

Figure 7



Bar diagrams representing results obtained when various stocks, as designated above, were cultured under axenic conditions on media containing whole casein with and without added ribonucleic acid. Black portion of bars represent results found on casein media lacking RNA; white portion of bars, those results obtained when RNA was present in the medium.

DISCUSSION

This investigation was begun with the expectation that blocks in biochemical reactions could be investigated from a genetic standpoint in Drosophila with a precision similar to that performed in analogous research with microorganisms. For a variety of reasons, that expectation has not been realized to date. Therefore, the following discussion is necessarily mainly concerned with the theoretical interpretations of the results actually obtained in this work.

The early experiments which utilized a synthetic medium containing amino acids obtained from British firms resulted in the complete failure of larval growth. This effect on growth might be explained on the assumption that traces of heavy metals (e.g., As, Ag, Pb etc.) were present as residual elements from the extraction processes used in the manufacture of large quantities of amino acids on a commercial scale. It must be further assumed that heavy metals are extremely toxic to Drosophila larvae feeding on a sterile synthetic medium; an assumption which is by no means a remote possibility when consideration is taken of the sub-optimal growth conditions already present.

As can be seen from the results presented in Table 3, the heavy metal concentration in any one amino acid could not be sufficient to cause complete larval mortality within fifteen

days following inoculation, since some larval growth and development occurred in each series. Complete toxicity was reached only when all 13 acids from British sources were present in the medium together.

Our experience with this medium has been presented here in some detail to prevent possible future misinterpretations of like results when isolated growth stimulatory substances for Drosophila are tested in media containing these acids as a nitrogen source.

In experiments designed to test which purine or pyrimidine moiety of RNA failed to be synthesized by Oregon-K larvae, entire sets had to be discarded due to infections that appeared in the experimental cultures. The data that were obtained, however, indicate that the synthesis of the pyrimidine riboside, cytidine, is limited in Oregon-K (Sang, personal communication, in earlier experiments also obtained evidence for a cytidine requirement in this stock). The median pupal and eclosion times were considerably shortened when the larvae were raised in the presence of this compound, and an appreciable increase in the number of pupae formed was also noted. The presence of either adenine or guanine in the medium decreased the median times to pupation and eclosion considerably, but failed to increase the number of pupae formed.

A reduction in the time spent in the larval stage, when the animals are raised on purine or pyrimidine supplemented

media (as described above), or on media deficient in RNA or its components (in experiments to be discussed below), is not considered sufficient evidence, in itself, for concluding that the nutritional requirement for these stocks has been fully met. Only when this effect is accompanied by a considerable increase in the number of pupae formed (as in the cytidine series) would such a conclusion be valid. The reasons for this interpretation of our results are presented in the following paragraphs.

Conditions for growth, which were met by the young larvae within the experimental cultures, varied from experiment to experiment. Improvements were produced from time to time in methods of handling the larvae during inoculations, and also were made in the composition of the various media used. These were done for the purpose of reducing early larval mortality and decreasing the time required for larval growth and development. With better handling techniques and with an improved medium which was, nonetheless, completely deficient in ribonucleic acid or its components, larval growth might be expected to proceed at a faster rate without resulting in an increase in the number that are able to achieve pupation. This would be true only provided some synthesis of the deficient compound was taking place within the larvae, providing them with sufficient quantities of the substance to meet their metabolic needs for growth.

It was observed during the course of all experiments performed, except the earliest ones, that some larvae (their numbers were, unfortunately, not recorded) continued to live on the media after all pupation had taken place. These non-pupating larvae, apparently, were unable to synthesize either a larger amount of RNA to meet the need for the process of pupation, or a specific substance which was needed for pupation and whose synthesis was dependent upon the prior formation of a threshold quantity of RNA.

When either adenine or guanine was supplied exogenously, Oregon-K larvae were able to synthesize enough RNA to permit a faster rate of growth and development to occur. Only a minority, however, were able to achieve sufficient synthesis to reach pupation. When cytidine was supplied synthesis was sufficient for both faster larval growth (median time 11.4 days) and a higher proportion of pupation (56%).

We were unable to find a specific chromosome locus, one gene, which was responsible for the inability of Oregon-K to synthesize its own nucleic acid from the ingredients of a nucleic acid-less medium. This fact alone does not constitute sufficient evidence of multigenic control over the synthesis of a component of nucleic acid. Possible crossing-over between the chromosome containing Moiré, and its homologue derived from Oregon-K, could eliminate such a gene from Stocks 3 and 6, provided, of course, the factor was located within

the third chromosome. The third chromosomes of Oregon-K were intentionally eliminated from Stocks 1, 2, 4 and 5 so that no evidence for the presence of the RNA requirement would be expected in these stocks if the gene was located there.

In each of the six stocks tested, the time spent in the larval stage was decreased from that found for Oregon-K alone, yet there occurred no appreciable increase in numbers of pupae formed (best seen in Figure 7). On the basis of the explanation presented above for this type of result, it must be concluded that all six stocks retained the requirement for RNA. If this is true, the nutritional anomaly may be under the control of several genes located throughout the chromosomal complement of Oregon-K so that some of them were present in all the stocks.

The unexpected growth obtained when In (2LR)40d/Cy sp^2 larvae, known to require adenine in their diet, were inoculated onto adenine-less casein medium containing a high concentration of hydrogen-ions, prompted further experiments to determine whether or not the requirement had been lost from the stock sometime prior to the test. The results obtained on an amino acid medium, similar to that used in establishing the adenine requirement, proved conclusively that under these latter nutritional conditions the requirement was still present in this stock and could be overcome by the addition of RNA. Our attention has been focused, therefore, on the

differences in composition between the two types of media in order to explain why growth would occur on one but not on the other.

The physical appearances of the two media were different; that containing the amino acids was a clear light-brown mixture whereas the casein medium at low pH appeared a whitish opaque mixture with a granular consistency largely due to the undissolved casein. At pH 7 and higher, the casein went into solution resulting in a medium not too dissimilar from the amino acid medium although considerably darker brown in color. The deep coloring was probably due to the caramelization of the sugar present in the medium when the latter was autoclaved. To test the possibility that the larvae were stimulated by the presence of macroscopic particles in their diet, an amino acid medium (Medium 3, Table 1) was prepared which contained particles of carbon black in a heavy suspension. The initial pH of this medium was 5.2. No growth or development of In (2LR)40d/Cy sp^2 larvae ensued after inoculation, indicating that the cause of larval growth at low pH on a casein medium lay elsewhere than in the particulate nature of that medium.

The vitamins, salts, sterols and sugar used in the two media were essentially the same (cf. Medium 3, Table 1 and Medium 3, Table 2) although their final concentrations differed. While whole casein contains amino acids in similar

proportions to those supplied in Medium 3 (Table 1), they are linked together through peptide bonds to form the protein. It is possible that the hydrolysis of casein, either by autoclaving in the presence of acid or by the action of the proteolytic enzymes in the larval gut, or both, may be only partial, yielding various peptides and free amino acids some of which were not supplied in the amino acid medium. Amongst these different products of hydrolysis, a compound might be present which participates in the synthesis of both the purine and the pyrimidine moieties of RNA. The existence of such a compound is necessary to explain the growth obtained with Oregon-K, a pyrimidine requirer, as well as with In (2LR)40d/Cy sp^2, a purine requirer, already mentioned in this discussion.

On first consideration, the problems involved in discovering such a compound in the constituents of casein hydrolysates seem almost insurmountable. Recent studies by Eagle (see Eagle 1955a, 1955b and 1955c), however, on the growth requirements of both neoplastic and normal cells on chemically defined media have focused our attention on the importance of glutamine, the acid amide of glutamic acid, in animal nutrition. Quite recently, Eagle has found glutamine to be essential for the growth of a number of different kinds of animal cells under tissue culture conditions (unpublished communication). This evidence, by itself, might not be par-

ticularly suggestive for our work were it not for the fact that glutamine is known to be implicated in a number of biochemical mechanisms through its ability to store and transport ammonia. It is known to enter into the synthesis of the purines in certain microorganisms and in pigeon liver (Greenberg 1954b). It is quite possible that glutamine plays a role in the formation of one or two of the precursors of the pyrimidine compounds, although the exact pathway leading to the synthesis of pyrimidines is much less understood at the present time than is that involving the synthesis of purines (see, however, Waelsch 1952 for a review of the intermediary metabolism of glutamine; Ratner 1954 for information concerning the role of glutamine in the synthesis of urea cycle compounds; and Heinrich, Dewey and Kidder 1954 for evidence for the incorporation of the carbamyl carbon of citrulline, a urea cycle compound, into carbon 2 of the pyrimidines in Neurospora).

Glutamine itself is formed from glutamic acid and ammonia in a reaction which is catalyzed by the enzyme, glutamine synthetase. The steps in this reaction are believed to be as follows:

$$\text{Enzyme} + \text{ATP} \rightleftharpoons \text{Enzyme} \sim \text{P} + \text{ADP}$$

$$\text{Enzyme} \sim \text{P} + \text{glutamic acid} \rightleftharpoons \text{Enzyme-glutamic acid} + \text{P}_i$$

$$\text{Enzyme-glutamic acid} + \text{NH}_3 \longrightarrow \text{Enzyme} + \text{glutamine}$$

It is hydrolyzed readily in the tissues through the action of

glutaminase to glutamic acid and ammonia. There are at least two glutaminases known to occur in animal tissues, one of which possesses a pH optimum between pH 7 and 8, the other an optimum between pH 8 and 9.

Our results, obtained when Oregon-K, In (2LR)40d/Cy sp^2 and Cy/Pm;D/Sb larvae were raised on nucleic acid-less casein media with low initial pH's, might be explicable if it is assumed that these larvae lack the ability to synthesize glutamine from glutamic acid and ammonia readily. It must be realized, of course, that other possibilities exist which may ultimately prove to be the cause of growth in such cultures, e.g., a requirement for the ribose moiety of RNA. Certain aspects of these alternative explanations, however, present greater difficulty, at least on theoretical grounds, than does the assumption that a requirement exists either for glutamine or a substance closely related to glutamine in the pathways of purine and pyrimidine synthesis.

The inability to synthesize glutamine may be a result of one or several causes. First, a direct cause would be that the larvae possess a partially inactivated glutamine synthetase for the catalysis of the synthetizing reaction. Second, the high level of glutamic acid (4.4 mg/ml) supplied in the media containing free amino acids, on which practically no growth occurred unless nucleic acid was also present, might not be sufficient for glutamine synthesis if the larval gut is

relatively impermeable to the acid. It is known that certain mammalian tissues (e.g., brain and kidney tissue) are more permeable to glutamine than to glutamic acid. Finally, the acidic conditions met by the larvae in nature, on ordinary laboratory media containing yeast, and in the casein media described here, might inactivate the action of glutaminase in the tissues so that threshold levels of glutamine necessary for larval growth and for pupation (see above) can be maintained. It is difficult, however, to visualize the inactivation of an enzyme in the tissues of the larvae when the animals are not bathed in an acidic fluid medium, but burrow inside a solid one.

There are organisms known which lack the ability to synthesize glutamine from glutamic acid. Streptococcus haemolyticus has been found to require glutamine for growth (McIlwain 1939); presumably it is unable to perform the reactions outlined above. Under certain conditions (i.e, large inocula), glutamic acid is able partially to replace glutamine, possibly through selection of genetically different organisms contained in the large inocula which are capable of utilizing glutamic acid (McIlwain, Fildes, Gladstone and Knight 1939). Under conditions where the requirement for glutamine is absolutely specific, however, S. haemolyticus was found to require glutamine solely for the initiation of growth, losing the requirement once growth had begun (Fildes and Gladstone 1939).

Pollack and Lindner (1942) investigated nine strains of lactic acid bacteria and found that five of them responded equally well to glutamic acid or glutamine. In the four remaining strains, however, at least eleven times as much glutamic acid as glutamine was required to produce equal growth. Lactobacillus arabinosus was found to be a glutamine-requirer by Hac, Snell and Williams (1945) who also observed that the "activity of (glutamic acid) is increased toward that of glutamine as a limit by increasing the size of the inoculum, lengthening the incubation period, lowering the initial pH of the medium, and adding ammonium salts to the medium.". These authors conclude that glutamic acid is converted first to glutamine before utilization by the organism. Lyman, Kuiken, Blotter and Hale (1945) found that when the glutamic acid concentration is low in the medium, L. arabinosus cannot readily carry out the conversion to glutamine.

At present, glutamine has not been tested in the media for Drosophila, so that the brief account of its functions in metabolic systems given above is necessarily of a speculative nature regarding its activity in Drosophila nutrition. Should this compound (or one related to it) prove to be active, it is obvious that In (2LR)40d/Cy sp^2, Oregon-K and Cy/Pm;D/Sb can no longer be considered "nucleic acid-requirers" as such, but should be classified as "glutamine-requirers". It is also possible, in view of the stimulation of growth observed when

nucleic acid is supplied to all Drosophila stocks so far tested under axenic conditions, that glutamine activity will result in enhanced larval growth and development more nearly approaching that achieved on a yeast diet for all Drosophila stocks raised on chemically defined media.

SUMMARY

Aspects of the nutrition of Drosophila melanogaster (Meigen) have been investigated, particularly the role of nucleic acid in stimulating growth and pupation of larvae raised under axenic conditions. Chemically defined and partially chemically defined media were used. Full descriptions of the techniques employed in establishing axenic cultures of Drosophila larvae are given.

No larval growth occurred on media containing 13 amino acids obtained from several British firms. It was suggested that traces of heavy metals as chemical contamination of these acids were responsible for the failure of larval growth.

Oregon-K, a phenotypically wild-type stock that was reported to have been inbred for over 700 generations, was found to require nucleic acid for growth, although some growth occurred in the absence of nucleic acid or its constituents. The pyrimidine riboside, cytidine, was found partially to replace the whole molecule of ribonucleic acid. Adenine and guanine, the purine bases, accelerated the rate of larval growth and development but failed to allow more pupae to be formed. The presence of the pyrimidine base, uracil, permitted no growth of the larvae whatsoever. This result was interpreted as due to the toxicity of the compound rather than due to a metabolic inactivity.

Attempts were made to localize on the chromosomes a genetic factor responsible for the requirement. All attempts failed to reveal the presence of such a single factor.

In further attempts to localize the gene, a mutant marker stock was used which grew well on nucleic acid-less casein medium, but did not on a nucleic acid-less medium containing free amino acids. These results led to an investigation of the effect on larval growth of varying hydrogen-ion concentrations in casein and amino acid media.
In (2LR)40d/Cy sp^2 and Oregon-K, known to require nucleic acid in their diets, were found able to grow well on nucleic acid-less casein media provided the hydrogen-ion concentration was high. In (2LR)40d/Cy sp^2 failed to grow under similar conditions, but with free amino acids substituted in the medium for casein.

Theoretical possibilities to explain the pH effect are presented and discussed. These suggest that the nucleic acid requirement in some Drosophila stocks may actually be a requirement for the acid amide of glutamic acid, glutamine, or some substance closely related to it, which would participate in purine and pyrimidine synthesis. It is further suggested that the presence of such a compound in a chemically defined medium would accelerate larval growth and development in all Drosophila stocks.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge my gratitude to Professor C. H. Waddington, F.R.S., Director of the Institute of Animal Genetics, University of Edinburgh, for offering me the hospitality and facilities of the Institute during my stay in Edinburgh, and for the sympathetic interest he has taken in this work. My thanks are also due Professor Waddington's staff for their many kindnesses extended to me.

Particular gratitude is owed Dr. James H. Sang, Principal Scientific Officer, Agricultural Research Council, for his just criticisms of my work and for the numerous instances that he offered constructive advice regarding it.

To my colleagues at the Biological Laboratories, Amherst College, and especially Professor G. W. Kidder, I owe thanks for their interest in this work, and for providing the necessary facilities to carry it out. It is also a pleasure to acknowledge the assistance of Dr. Philip T. Ives, who read most of the manuscript of this thesis and who offered useful advice concerning its contents.

LITERATURE CITED

ALPATOV, W. W., 1932 Egg production in Drosophila melanogaster and some factors which influence it. Jour. Exp. Zool. 63:85-111.

BACOT, A. W., and A. HARDEN, 1922 Vitamin requirements of Drosophila. I. Vitamins B and C. Biochem. Jour. 16: 148-152.

BAKER, J. A., and M. S. FERGUSON, 1942 Growth of platyfish (Platypoecilus maculatus) free from bacteria and other microorganisms. Proc. Soc. Exp. Biol. Med. 51:116-119.

BARRATT, R. W., D. NEWMEYER, D. D. PERKINS and L. GARNJOBST, "Map construction in Neurospora crassa", pp 1-93, in: Advances in Genetics, Vol. VI, ed., M. Demerec. Academic Press, Inc. New York.

BATESON, W., and E. R. SAUNDERS, 1902 Report of the Evolution Committee of the Royal Society, No. 1. pp 133-134. Note.

BAUMBERGER, J. P., 1917a Solid media for rearing Drosophila. Amer. Nat. 51:447-448.

BAUMBERGER, J. P., 1917b The food of Drosophila melanogaster Meigen. Proc. Nat. Acad. Sci. 3:122-126.

BAUMBERGER, J. P., 1919 A nutritional study of insects with special reference to microorganisms and their substrata. Jour. Exp. Zool. 28:1-81 (Spanish summary).

BAUMBERGER, J. P., and R. W. GLASER, 1917 The rearing of Drosophila ampelophila Loew on solid media. Science 45: 21-22.

BEADLE, G. W., 1945 Biochemical genetics. Chem. Rev. 37: 15-96.

BEADLE, G. W., 1946 Genes and the chemistry of the organism. Amer. Sci. 34:31-53 and 76.

BEADLE, G. W., 1951 "Chemical genetics", pp 221-239, in: Genetics in the 20th Century, ed., L. C. Dunn. The Macmillan Co. New York.

BEADLE, G. W., and B. EPHRUSSI, 1936 The differentiation of eye pigments in Drosophila as studied by transplantation. Genetics 21:225-247.

BEADLE, G. W., R. L. ANDERSON and J. MAXWELL, 1938 A comparison of the diffusible substances concerned with eye color development in Drosophila, Ephestia and Habrobracon. Proc. Nat. Acad. Sci. 24:80-84.

BEADLE, G. W., and L. W. LAW, 1938 Influence on eye color of feeding diffusible substances to Drosophila melanogaster. Proc. Soc. Exp. Biol. 37:621-623.

BEADLE, G. W., and E. L. TATUM, 1941 Genetic control of biochemical reactions in Neurospora. Proc. Nat. Acad. Sci. 27:499-506.

BEADLE, G. W., H. K. MITCHELL and J. F. NYC, 1947 Kynurenine as an intermediate in the formation of nicotinic acid from tryptophane by Neurospora. Proc. Nat. Acad. Sci. 33:155-158.

BEGG, M., 1949 Nutritional requirements of Drosophila. Nature 163:881.

BEGG, M., and F. W. ROBERTSON, 1948 Nutritional requirements of Drosophila melanogaster. Nature 161:769-770.

BEGG, M., and F. W. ROBERTSON, 1950 The nutritional requirements of Drosophila melanogaster. Jour. Exp. Biol. 26: 380-387.

BEGG, M., and J. H. SANG, 1950 A method for collecting and sterilizing large numbers of Drosophila eggs. Science 112:11-12.

BENZER, S., 1955 Fine structure of a genetic region in bacteriophage. Proc. Nat. Acad. Sci. 41:344-354.

BÖDEKER, C. H. D., 1859 Ueber das Alcapton; ein neuer Beitrag zur Frage: welche Stoffe des Harns können Kupferreduction bewirken? Ztschr. f. rat. Med. (third series) 7:130-145.

BONNER, D., 1946a Production of biochemical mutations in Penicillium. Amer. Jour. Bot. 33:788-791.

BONNER, D., 1946b Biochemical mutations in Neurospora. Cold Spring Harbor Symposia on Quant. Biol. 11:14-24.

BONNER, D., 1948 The identification of a natural precursor of nicotinic acid. Proc. Nat. Acad. Sci. 34:5-9.

BONNER, D. M., 1952 "The genetic control of enzyme formation", pp 153-166, in: A Symposium on Phosphorus Metabolism, Vol. II, eds., W. D. McElroy and B. Glass. The Johns Hopkins Press. Baltimore.

BONNER, D., and G. W. BEADLE, 1946 Mutant strains of Neurospora requiring nicotinamide or related compounds for growth. Arch. Biochem. 11:319-328.

BONNER, D., and C. YANOFSKY, 1949 Quinolinic acid accumulation in the conversion of 3-hydroxyanthranilic acid to niacin in Neurospora. Proc. Nat. Acad. Sci. 35:576-581.

BONNER, D. M., C. YANOFSKY and C. W. H. PARTRIDGE, 1952 Incomplete genetic blocks in biochemical mutants of Neurospora. Proc. Nat. Acad. Sci. 38:25-34.

BRIDGES, C. B., 1935 Salivary chromosome maps. With a key to the banding of the chromosomes of Drosophila melanogaster. Jour. Hered. 26:60-64.

BRUST, M., and G. FRAENKEL, 1955 The nutritional requirements of the larvae of a blowfly, Phormia regina (Meig.). Physiol. Zool. 28:186-204.

BUTENANDT, A., W. WEIDEL and E. BECKER, 1940 Kynurenin als Augenpigmentbildung auslosendes Agens bei Insekten. Naturwiss. 28:63-64.

BUTENANDT, A., W. WEIDEL and H. SCHLOSSBERGER, 1949 3-oxykynurenin als cn^{+}-Genabhangiges Glied im intermediaren Tryptophan-Stoffwechsel. Z. Naturforsch. 46: 242-244.

CASPARI, E., 1933 Über die Wirkung eines pleiotropen Gens bei der Mehlmotte Ephestia kühniella Zeller. Arch. Entw. Org. 130:353-381.

CASPARI, E., 1949 Physiological action of eye color mutants in the moths Ephestia kühniella and Ptychopoda seriata. Quart. Rev. Biol. 24:185-199.

CHARGAFF, E., 1955 "Isolation and composition of the deoxypentose nucleic acids and of the corresponding nucleoproteins", pp 307-371, in: The Nucleic Acids, eds., E. Chargaff and J. N. Davidson. Academic Press, Inc. New York.

CHARGAFF, E., S. ZAMENHOF, G. BRAWERMAN and L. KERIN, 1950 Bacterial deoxypentose nucleic acids of unusual composition. Jour. Amer. Chem. Soc. 72:3825.

COMMONER, B., 1949 On the interpretation of the absorption of ultraviolet light by cellular nucleic acids. Science 110:31-40.

DARWIN, C., 1868 The Variation of Animals and Plants under Domestication. Authorized Amer. Ed. Vol. II, Ch. XVII. Orange Judd and Co. New York.

DELCOURT, A., and E. GUYÉNOT, 1911 Genétique et milieu. Necessité de la détermination des conditions. Sa possibilité chez Drosophiles. Technique. Bull. Sci. France et Belgique 45:249-332.

DEMEREC, M., 1955 What is a gene? - Twenty years later. Amer. Nat. 89:5-20.

DEMEREC, M., I. BLOMSTRAND and Z. E. DEMEREC, 1955 Evidence of complex loci in Salmonella. Proc. Nat. Acad. Sci. 41:359-364.

DOBZHANSKY, T., 1941 Genetics and the Origin of Species. Columbia Univ. Press. New York. 2nd Ed. pp i-xvii + 1-446.

DODGE, B. O., 1927 Nuclear phenomena associated with heterothallism and homothallism in the ascomycete Neurospora. Jour. Agric. Rev. 35:289-305.

EAGLE, H., 1955a The specific amino acid requirements of a mammalian cell (Strain L) in tissue culture. Jour. Biol. Chem. 214:839-852 + 1 plate.

EAGLE, H., 1955b Utilization of dipeptides by mammalian cells in tissue culture. Proc. Soc. Exp. Biol. Med. 89: 96-99.

EAGLE, H., 1955c The specific amino acid requirements of a human carcinoma cell (Strain HeLa) in tissue culture. Jour. Exp. Med. 102:37-48 + 4 plates.

ELLIOTT, A. M., and D. F. GRUCHY, 1952 The occurrence of mating types in Tetrahymena. Biol. Bull. 103:301.

ELLIOTT, A. M., and R. E. HAYES, 1953 Mating types in Tetrahymena. Biol. Bull. 105:269-284.

ELLIS, J. F., 1950 Purines and pyrimidines in the nutrition of Drosophila. Unpublished Master's Thesis. Amherst College, Amherst, Mass. USA.

EPHRUSSI, B., 1942 Chemistry of "eye color hormones" of Drosophila. Quart. Rev. Biol. 17:327-338.

EPHRUSSI, B., and G. W. BEADLE, 1935 La transplantation des disques imaginaux chez la Drosophile. C. R. Acad. Sci. Paris. 201:98-100.

EPHRUSSI, B., and G. W. BEADLE, 1936 A technique of transplantation for Drosophila. Amer. Nat. 70:218-225.

FALTA, W., 1904 Der Eiweisstoffwechsel bei der Alkaptonurie. Dtsch. Arch. klin. Med. 81:231-277.

FILDES, P., and G. P. GLADSTONE, 1939 Glutamine and the growth of bacteria. Brit. Jour. Exp. Path. 20:334-341.

FLETCHER, W. E., J. M. GULLAND, D. O. JORDAN and in (part) H. E. DIBBEN, 1944 The constitution of yeast ribonucleic acid. Part VII. Diffusion coefficients and molecular weights. Jour. Chem. Soc. 1944:30-33.

FÖLLING, A., 1934 Über Ausscheidung von Phenylbrenztraubensäure in den Harn als Stoffwechselanomalie in Verbindung mit Imbezillität. Hoppe-Seyl. Ztschr. f. physiol. Chem. 227:169-176.

FRAENKEL, G. S., S. FRIEDMAN, T. HINTON, S. LASZLO and J. NOLAND, 1955 The effect of substituting carnitine for choline in the nutrition of several organisms. Arch. Biochem. and Biophys. 54:432-439.

GAMOW, G., 1954 Possible relation between deoxyribonucleic acid and protein structure. Nature 173:318.

GARROD, A. E., 1902 The incidence of alkaptonuria: a study in chemical individuality. Lancet 2:1616-1620.

GARROD, A. E., 1909 Inborn Errors of Metabolism. 1st Ed., Oxford Univ. Press. London and New York.

GARROD, A. E., 1923 Inborn Errors of Metabolism. 2nd Ed., H. Frowde and Hodder and Stoughton. London. and Oxford Univ. Press. London and New York. pp 1-216.

GARROD, A. E., 1931 The Inborn Factors of Disease. An Essay. Oxford Univ. Press. London and New York. pp 1-160.

GOLDSCHMIDT, R., 1916 Genetic factors and enzyme reaction. Science 43:98-100.

GOLDSCHMIDT, R., 1927 Physiologische Theorie der Vererbung. I. Springer. Berlin. pp 1-246.

GOLDSCHMIDT, R., 1938 Physiological Genetics. McGraw-Hill Book Co., Inc. New York. pp i-x + 1-375.

GORDON, C., and J. H. SANG, 1941 The relation between nutrition and exhibition of the gene 'antennaless' (Drosophila melanogaster). Proc. Roy. Soc. B, 130: 151-184.

GREEN, M. M., 1949 A study of tryptophane in eye color mutants of Drosophila. Genetics 34:564-572.

GREEN, M. M., 1952 Mutant isoalleles at the vermilion locus in Drosophila melanogaster. Proc. Nat. Acad. Sci. 38: 300-305.

GREEN, M. M., 1954 Pseudo-allelism at the vermilion locus in Drosophila melanogaster. Proc. Nat. Acad. Sci. 40:92-99.

GREEN, M. M., 1955 Homologous eye color mutants in the honeybee and Drosophila. Evol. 9:215-216.

GREENBERG, D. M., 1954a "Carbon catabolism of amino acids", pp 47-112, in: Chemical Pathways of Metabolism, Vol. II, ed., D. M. Greenberg. Academic Press, Inc. New York.

GREENBERG, G. R., 1954b Role of folic acid derivatives in purine biosynthesis. Fed. Proc. 13:745-759.

GROSS, O., 1914 Über den Einfluss des Blutserums des Normalen und des Alkaptonurikers auf Homogentisinsäure. Biochem. Z. 61:165-170.

GRÜNEBERG, H., 1938 An analysis of the "pleiotropic" effects of a new lethal mutation in the rat (Mus norvegicus). Proc. Roy. Soc. B, 125:123-144.

GUYÉNOT, E., 1913 Études biologiques sur une mouche, Drosophila ampelophila Löw. II. Role des leuvres dans l'alimentation. C. R. Soc. Biol. Paris. 74:178-180.

HAC, L. R., E. E. SNELL and R. J. WILLIAMS, 1945 The microbiological determination of amino acids. II. Assay and utilization of glutamic acid and glutamine by Lactobacillus arabinosus. Jour. Biol. Chem. 159:273-289.

HALDANE, J. B. S., 1935 Contributions de la génétique à la solution de quelques problèmes physiologiques. C. R. Soc. Biol. Paris. 119:1481-1496.

HALDANE, J. B. S., 1937 "The biochemistry of the individual", pp 1-10, in: Perspectives in Biochemistry, eds., J. Needham and D. E. Green. Cambridge Univ. Press. London and New York.

HALDANE, J. B. S., 1942 New Paths in Genetics. Harper and Brothers. New York and London. pp 1-206.

HALDANE, J. B. S., 1954 The Biochemistry of Genetics. George Allen and Unwin, Ltd. London. pp 1-144.

HARRIS, H., 1953 An introduction to human biochemical genetics. Eugenics Laboratory Memoirs, XXXVII. Cambridge Univ. Press. London.

HASSETT, C. C., 1948 The utilization of sugars and other substances by Drosophila. Biol. Bull. 95:114-123.

HEINRICH, M. R., V. C. DEWEY and G. W. KIDDER, 1954 Citrulline as a precursor of pyrimidines. Jour. Amer. Chem. Soc. 76:3102.

HIER, S. W., 1947 Influence of ingestion of single amino acids on the blood level of five amino acids. Jour. Biol. Chem. 171:813-820.

HINTON, T., 1947 Factors influencing the expression of "position effects". Biol. Bull. 93:216 (abstr.).

HINTON, T., 1948a Analysis of chromosomal rearrangements affecting the expression of a position-effect. Rec. Gen. Soc. Amer. 17:40 (abstr.).

HINTON, T., 1948b An analysis of rearrangements of chromosomal aberrations producing position effects. Genetics 33:108 (abstr.).

HINTON, T., 1949a The modification of the expression of a position effect. Amer. Nat. 83:69-94.

HINTON, T., 1949b The role of heterochromatin in position effect. Proc. 8th Inter. Cong. Genetics pp 595-596.

HINTON, T., 1950 A correlation of phenotypic changes and chromosomal rearrangements at the two ends of an inversion. Genetics 35:188-205.

HINTON, T., 1955 The genetic basis of a nutritional requirement in Drosophila. Genetics 40:224-234.

HINTON, T., and K. C. ATWOOD, 1941 "Mottleds in the Second Chromosome", p 231, in: The Gene by M. Demerec, B. P. Kaufmann, E. Sutton and U. Fano. Carnegie Inst. Wash. Year Book 40:225-234.

HINTON, T., J. ELLIS and D. T. NOYES, 1951 An adenine requirement in a strain of Drosophila. Proc. Nat. Acad. Sci. 37:293-299.

HINTON, T., D. T. NOYES and J. ELLIS, 1951 Amino acids and growth factors in a chemically defined medium for Drosophila. Physiol. Zool. 24:335-353.

HOGBEN, L., 1939 Nature and Nurture. Rev. Ed. W. W. Norton and Co., Inc. New York. pp 1-144.

HOGBEN, L., R. L. WORRALL and I. ZIEVE, 1932 The genetic basis of alkaptonuria. Proc. Roy. Soc. Edin. 52:264-295.

HOOG, E. G. van't, 1935a Aseptic culture of insects in vitamin research. Ztschr. f. Vitaminforsch. 4:300-323.

HOOG, E. G. van't, 1935b Symbiontless culture of Drosophila as a test-object on deficient food. Acta Brevia Neerland. 5:16-18.

HOOG, E. G. van't, 1936 Aseptic culture of insects in vitamin research (continued). Ztschr. f. Vitaminforsch. 5:118-125.

HOROWITZ, N. H., 1950 "Biochemical genetics of Neurospora", pp 33-71, in: Advances in Genetics, Vol. III, ed., M. Demerec. Academic Press, Inc. New York.

HOROWITZ, N. H., and U. LEUPOLD, 1951 Some recent studies bearing on the one gene - one enzyme hypothesis. Cold Spring Harbor Symposia on Quant. Biol. 16:65-74.

HOUSE, H. L., 1954a Nutritional studies with Pseudosarcophaga affinis (Fall.), a dipterous parasite of the spruce budworm, Choristoneura fumiferana (Clem.). I. A chemically defined medium and aseptic-culture technique. Canadian Jour. Zool. 32:331-341.

HOUSE, H. L., 1954b Nutritional studies with Pseudosarcophaga affinis (Fall.), a dipterous parasite of the spruce budworm, Choristoneura fumiferana (Clem.). II. Effects of eleven vitamins on growth. Canadian Jour. Zool. 32:342-350.

HOUSE, H. L., 1954c Nutritional studies with Pseudosarcophaga affinis (Fall.), a dipterous parasite of the spruce budworm, Choristoneura fumiferana (Clem.). III. Effects of nineteen amino acids on growth. Canadian Jour. Zool. 32:351-357.

HOUSE, H. L., 1954d Nutritional studies with Pseudosarcophaga affinis (Fall.), a dipterous parasite of the spruce budworm, Choristoneura fumiferana (Clem.). IV. Effects of ribonucleic acid, glutathione, dextrose, a salt mixture, cholesterol and fats. Canadian Jour. Zool. 32:358-365.

HOWLAND, R. B., E. A. GLANCY and B. P. SONNENBLICK, 1937 Transplantation of wild type and vermilion eye disks among four species of Drosophila. Genetics 22:434-442.

IRWIN, M. R., 1951 "Genetics and Immunology", pp 173-219, in: Genetics in the 20th Century, ed., L. C. Dunn. The Macmillan Co. New York.

IVES, P. T., 1939 The effects of high temperature on bristle frequencies in scute and wild-type males of Drosophila melanogaster. Genetics 24:315-331.

KHOUVINE, V., B. EPHRUSSI and S. CHEVAIS, 1938 Development of eye colors in Drosophila: Effect of yeast, peptones and starvation on their production. Biol. Bull. 75:425-445.

KIDDER, G. W., 1953 "The nutrition of invertebrate animals", pp 162-196, in: Biochemistry and Physiology of Metabolism, eds., G. H. Bourne and G. W. Kidder. Academic Press, Inc. New York.

KIDDER, G. W., and V. C. DEWEY, 1951 "The biochemistry of ciliates in pure culture", pp 323-400, in: Biochemistry and Physiology of Protozoa, ed., A. Lwoff. Academic Press, Inc. New York.

KIKKAWA, H., 1941 Mechanism of pigment formation in Bombyx and Drosophila. Genetics 26:587-607.

KIKKAWA, H., 1953 "Biochemical genetics of Bombyx mori (Silkworm)", pp 107-140, in: Advances in Genetics, Vol. V, ed., M. Demerec. Academic Press, Inc. New York.

KNOX, W. E., and A. H. MEHLER, 1950 The conversion of tryptophan to kynurenine in liver. I. The coupled tryptophan peroxidase-oxidase system forming formyl-kynurenine. Jour. Biol. Chem. 187:419-430.

LA DU, Jr., B. N., and D. M. GREENBERG, 1951 The tyrosine oxidation system of liver. 1. Extracts of rat liver acetone powder. Jour. Biol. Chem. 190:245-255.

LAFON, M., 1937 Les facteurs liposolubles nécessaires à la croissance de Drosophila melanogaster Meig. C. R. Soc. Biol. Paris. 124:798-800.

LAFON, M., 1938 Le besoin qualitatif d'azote chez Drosophila melanogaster Meig. C. R. Acad. Sci. Paris. 207:306-308.

LAUGHNAN, J. R., 1948 The action of allelic forms of the gene A in maize. I. Studies of variability, dosage and dominance relations. The divergent character of the series. Genetics 33:488-517.

LAUGHNAN, J. R., 1949 The action of allelic forms of the gene A in maize. II. The relation of crossing over to mutation of A^b. Proc. Nat. Acad. Sci. 35:167-178.

LAUGHNAN, J. R., 1950 The action of allelic forms of the gene A in maize. III. Studies on the occurrence of isoquercitrin in brown and purple plants and its lack of identity with the brown pigments. Proc. Nat. Acad. Sci. 36:312-318.

LAUGHNAN, J. R., 1952 The action of allelic forms of the gene A in maize. IV. On the compound nature of A^b and the occurrence and action of its A^d derivatives. Genetics 37:375-395.

LAUGHNAN, J. R., 1955 Structural and functional bases for the action of the A alleles in maize. Amer. Nat. 89: 91-103.

LEAF, G., and A. NEUBERGER, 1948 The preparation of homogentisic acid and of 2:5-dihydroxyphenylethylamine. Biochem. Jour. 43:606-610.

LEDERBERG, J., 1951 "Genetic studies with bacteria", pp 263-289, in: Genetics in the 20th Century, ed., L. C. Dunn. The Macmillan Co. New York.

LEWIS, E. B., 1955 Some aspects of position pseudoallelism. Amer. Nat. 89:73-89.

LINDEGREN, C. C., 1932a The genetics of Neurospora. I. The inheritance of response to heat treatment. Bull. Torrey Bot. Club 59:85-102.

LINDEGREN, C. C., 1932b The genetics of Neurospora. II. Segregation of the sex factor in asci of N. crassa, N. sitophila and N. tetrasperma. Bull. Torrey Bot. Club 59:119-138.

LINDEGREN, C. C., 1933 The genetics of Neurospora. III. Pure bred stocks and crossing over in N. crassa. Bull. Torrey Bot. Club 60:133-154.

LOEB, J., 1915a The simplest constituents required for growth and the completion of the life cycle in an insect (Drosophila). Science 41:169-170.

LOEB, J., 1915b The salts required for the development of insects. Jour. Biol. Chem. 23:431-434.

LOEB, J., and J. H. NORTHROP, 1916 Nutrition and evolution, second note. Jour. Biol. Chem. 27:309-312.

LWOFF, A., 1946 Some problems connected with spontaneous biochemical mutations in bacteria. Cold Spring Harbor Symposia on Quant. Biol. 11:139-155.

LYMAN, C. M., K. A. KUIKEN, L. BLOTTER and F. HALE, 1945 The microbiological determination of amino acids. II. Glutamic acid. Jour. Biol. Chem. 157:395-405.

MACKENDRICK, M. E., and G. PONTECORVO, 1952 Crossing over between alleles at the w locus in Drosophila melanogaster. Experientia 8:390-391.

MARSHALL, J., 1887 A preliminary notice of a crystalline acid in urine possessing more powerful reducing properties than glucose. Medical News (Philadelphia) 1:35-37.

MC CLINTOCK, B., 1951 Chromosome organization and genic expression. Cold Spring Harbor Symposia on Quant. Biol. 16:13-47.

MC ILWAIN, H., 1939 The specificity of glutamine for growth of Streptococcus haemolyticus. Biochem. Jour. 33: 1942-1946.

MC ILWAIN, H., 1946 The magnitude of microbial reactions involving vitamin-like compounds. Nature 158:898-902.

MC ILWAIN, H., P. FILDES, G. P. GLADSTONE and B. C. J. G. KNIGHT, 1939 Glutamine and the growth of Streptococcus haemolyticus. Biochem. Jour. 33:223-229.

MEDES, G., 1932 A new error of tyrosine metabolism: tyrosinosis; the intermediary metabolism of tyrosine and phenylalanine. Biochem. Jour. 26:917-940.

MEHLER, A. H., and W. E. KNOX, 1950 The conversion of tryptophan to kynurenine in liver. II. The enzymatic hydrolysis of formylkynurenine. Jour. Biol. Chem. 187:431-438.

MITCHELL, H. K., and M. B. HOULAHAN, 1946a Neurospora. IV. A temperature sensitive riboflavinless mutant. Amer. Jour. Bot. 33:31-35.

MITCHELL, H. K., and M. B. HOULAHAN, 1946b Adenine requiring mutants of Neurospora crassa. Fed. Proc. 5:370-375.

MITCHELL, H. K., and J. LEIN, 1948 A Neurospora mutant deficient in the enzymatic synthesis of tryptophan. Jour. Biol. Chem. 175:481-482.

MITCHELL, H. K., and J. F. NYC, 1948 Hydroxyanthranilic acid as a precursor of nicotinic acid in Neurospora. Proc. Nat. Acad. Sci. 34:1-5.

MONOD, J., 1947 The phenomenon of enzymatic adaptation and its bearings on problems of genetics and cellular differentiation. Growth 11:223-289.

MOORE, A. R., 1910 A biochemical conception of dominance. Univ. Calif. Publ. in Physiol. 4:9-15.

MORGAN, T. H., 1926 Genetics and the physiology of development. Amer. Nat. 60:489-515.

MOSS, A. R., and R. SCHOENHEIMER, 1940 The conversion of phenylalanine to tyrosine in normal rats. Jour. Biol. Chem. 135:415-429.

MULLER, H. J., 1941 Résumé and perspectives of the symposium on genes and chromosomes. Cold Spring Harbor Symposia on Quant. Biol. 9:290-308.

NANNEY, D. L., 1953 Nucleo-cytoplasmic interaction during conjugation in Tetrahymena. Biol. Bull. 105:133-148.

NANNEY, D. L., and P. A. CAUGHEY, 1953 Mating type determination in Tetrahymena pyriformis. Proc. Nat. Acad. Sci. 39:1057-1063.

NANNEY, D. L., and P. A. CAUGHEY, 1955 An unstable nuclear condition in Tetrahymena pyriformis. Genetics 40: 388-398.

NEEL, J. V., and W. J. SCHULL, 1954 Human Heredity. Univ. Chicago Press. Chicago. pp 1-361.

NENCKI, M., 1886 Bemerkungen zu einer Bemerkung Pasteur's. Arch. Exp. Path. 20:385-388.

NEUBAUER, O., and W. FALTA 1904 Über das Schicksal einiger aromatischer Säuren bei der Alkaptonurie. Ztschr. f. physiol. Chem. 42:81-101.

NORTHROP, J. H., 1917 The role of yeast in the nutrition of an insect (Drosophila). Jour. Biol. Chem. 30:181-187.

PASTEUR, L., 1885 Observations relatives à la note précédente de M. Duclaux. C. R. des Séances de l'academie des Sciences 100:68.

PAULING, L., and R. B. COREY, 1953a Structure of the nucleic acids. Nature 171:346.

PAULING, L., and R. B. COREY, 1953b A proposed structure for the nucleic acids. Proc. Nat. Acad. Sci. 39:84-97.

PLAINE, H. L., and B. GLASS, 1955 Influence of tryptophan and related compounds upon the action of a specific gene and the induction of melanotic tumours in Drosophila melanogaster (with two text figures). Jour. Genetics 53:244-261.

PLUNKETT, C. R., 1926 The interaction of genetic and environmental factors in development. Jour. Exp. Zool. 46: 181-244.

PLUNKETT, C. R., 1932 Temperature as a tool of research in phenogenetics: methods and results. Proc. 6th Int. Cong. Genetics 2:158-160.

POLLACK, M. A., and M. LINDNER, 1942 Glutamine and glutamic acid as growth factors for lactic acid bacteria. Jour. Biol. Chem. 143:655-661.

PONTECORVO, G., J. A. ROPER, L. M. HEMMONS, K. D. MACDONALD and A. W. J. BUFTON, 1953 "The genetics of Aspergillus nidulans", pp 141-238, in: Advances in Genetics, Vol. V, ed., M. Demerec. Academic Press, Inc. New York.

POWSNER, L., 1935 The effects of temperature on the duration of the developmental stages of Drosophila melanogaster. Physiol. Zool. 8:474-520.

PRICE, Jr., J. B., 1949 IV. Transplantation experiments in Drosophila virilis: The formation of brown pigment. Univ. Texas Publ. No. 4920:24-30.

RATNER, S., 1954 "Urea synthesis and metabolism of arginine and citrulline", pp 319-387, in: Advances in Enzymology, Vol. XV, ed., F. F. Nord. Interscience Publishers, Inc. New York.

RAVDIN, R. G., and D. I. CRANDALL, 1951 The enzymatic conversion of homogentisic acid to 4-fumarylacetoacetic acid. Jour. Biol. Chem. 189:137-149.

ROBERTSON, F. W., and J. H. SANG, 1944 The ecological determinants of population growth in a Drosophila culture. I. Fecundity of adult flies. Proc. Roy. Soc. B, 132: 258-277.

ROPER, J. A., 1950 Search for linkage between genes determining a vitamin requirement. Nature 166:956-957.

ROSE, W. C., W. J. HAINES, J. E. JOHNSON and D. T. WARNER, 1943 Further experiments on the role of the amino acids in human nutrition. Jour. Biol. Chem. 148:457-458.

RUDKIN, G. T., and J. SCHULTZ, 1947 Evolution of nutritional requirements in animals: amino acids essential for Drosophila melanogaster. Anat. Rec. 99:613 (abstr.).

RUDKIN, G. T., and J. SCHULTZ, 1949 A comparison of the tryptophane requirements of mutant and wild type Drosophila melanogaster. Proc. 8th Int. Cong.

Genetics. (issued as a supplementary volume of Hereditas) pp 652-653 (abstr.).

SANG, J. H., 1949a The ecological determinants of population growth in a Drosophila culture. III. Larval and pupal survival. Physiol. Zool. 22:183-202.

SANG, J. H., 1949b The ecological determinants of population growth in a Drosophila culture. IV. The significance of successive batches of larvae. Physiol. Zool. 22:202-210.

SANG, J. H., 1949c The ecological determinants of population growth in a Drosophila culture. V. The adult population count. Physiol. Zool. 22:210-223.

SANG, J. H., 1950 Population growth in Drosophila cultures. Biol. Rev. Cambridge Phil. Soc. 25:188-219.

SANG, J. H., J. M. MCDONALD and C. GORDON, 1949 The ecological determinants of population growth in a Drosophila culture. Physiol. Zool. 22:223-235.

SANG, J. H., and J. M. MCDONALD, 1954 Production of phenocopies in Drosophila using salts, particularly sodium metaborate. Jour. Genetics 52:392-412.

SCHULTZ, J., 1936 Variegation in Drosophila and the inert chromosome regions. Proc. Nat. Acad. Sci. 22:27-33.

SCHULTZ, J., 1941a The function of heterochromatin. Proc. 7th Int. Cong. Genetics pp 257-262.

SCHULTZ, J., 1941b The evidence of the nucleoprotein nature of the gene. Cold Spring Harbor Symposia on Quant. Biol. 9:55-65.

SCHULTZ, J., 1947 The nature of heterochromatin. Cold Spring Harbor Symposia on Quant. Biol. 12:179-191.

SCHULTZ, J., and T. CASPERSSON, 1939 Heterochromatic regions and the nucleic acid metabolism of the chromosomes. Arch. Zellforsch. 22:650-654.

SCHULTZ, J., T. CASPERSSON and L. AQUILONIUS, 1940 The genetic control of nucleolar composition. Proc. Nat. Acad. Sci. 26:515-523.

SCHULTZ, J., P. ST. LAWRENCE and D. NEWMEYER, 1946 A chemically defined medium for the growth of Drosophila melanogaster. Anat. Rec. 96:540 (abstr.).

SCHULTZ, J., and G. T. RUDKIN, 1948 Absence of a sparing action of tryptophan on nicotinamide requirements of the fly, Drosophila melanogaster. Fed. Prod. 7:185 (abstr.).

SCHULTZ, J., and G. T. RUDKIN, 1949 Nutritional requirements and the chemical genetics of Drosophila melanogaster. Proc. 8th Int. Cong. Genetics. (issued as a supplementary volume of Hereditas) pp 657-658 (abstr.).

SCHULTZ, J., and M. M. SERVICE, 1951 Genetic differences in the requirement for ribosenucleic acid and glycine in Drosophila melanogaster. Fed. Proc. 10:245 (abstr.).

SCHWARTZ, D., 1955 Speculations on gene action and protein specificity. Proc. Nat. Acad. Sci. 41:300-307.

SCRIBONIUS, G. A., 1584 De Inspectione Urinarum (cited by Garrod 1923).

SPENCER, W. P., 1950 "Collection and Laboratory Culture", pp 535-590, in: Biology of Drosophila, ed., M. Demerec. John Wiley and Sons, Inc. New York.

SPRITES, M. A., 1951 Demonstration of the presence of Krebs cycle enzymes in Drosophila melanogaster. Fed. Proc. 10:251 (abstr.).

STADLER, L. J., 1951 Spontaneous mutation in maize. Cold Spring Harbor Symposia on Quant. Biol. 16:49-63.

STOKES, J. L., J. W. FOSTER and C. R. WOODWARD Jr., 1946 Synthesis of pyridoxin by a "pyridoxinless" x-ray mutant of Neurospora sitophila. Arch. Biochem. 2:235-245.

STRAUSS, B. S., 1951 Studies on the B_6-requiring, pH sensitive mutants of Neurospora crassa. Arch. Biochem. 30: 292-305.

STURTEVANT, A. H., 1920 The vermilion gene and gynandromorphism. Proc. Soc. Exp. Biol. Med. 17:70-71.

STURTEVANT, A. H., 1932 The use of mosaics in the study of developmental effects of genes. Proc. 6th Int. Cong. Genetics 2:304-307.

STURTEVANT, A. H., and E. NOVITSKI, 1941 The homologies of the chromosome elements in the genus Drosophila. Genetics 26:517-541.

SUDA, M., and Y. TAKEDA, 1950 Metabolism of tyrosine. II. Homogentisicase. Jour. Biochem. (Japan) 37:381-384.

TAN, C. C., and D. F. POULSON, 1937 The behavior of vermilion and orange eye colors in transplantation in Drosophila pseudoobscura. Jour. Genetics 34:433-435.

TATUM, E. L., 1939a Development of eye-colors in Drosophila: Bacterial synthesis of v^+ hormone. Proc. Nat. Acad. Sci. 25:486-490.

TATUM, E. L., 1939b Nutritional requirements of Drosophila melanogaster. Proc. Nat. Acad. Sci. 25:490-497.

TATUM, E. L., 1941 Vitamin B requirements of Drosophila melanogaster. Proc. Nat. Acad. Sci. 27:193-197.

TATUM, E. L., 1949 Amino acid metabolism in mutant strains of microorganisms. Fed. Proc. 8:511-517.

TATUM, E. L., 1951 "Genetic aspects of growth responses in fungi", pp 447-461, in: Plant Growth Substances, ed., F. Skoog. Univ. Wisconsin Press. Madison.

TATUM, E. L., and G. W. BEADLE, 1940 Crystalline Drosophila eye-color hormone. Science 91:458.

TATUM, E. L., and A. J. HAAGEN-SMIT, 1941 Identification of Drosophila v^+ hormone of bacterial origin. Jour. Biol. Chem. 140:575-580.

TATUM, E. L., and D. BONNER, 1944 Indole and serine in the biosynthesis and breakdown of tryptophane. Proc. Nat. Acad. Sci. 30:30-37.

TATUM, E. L., S. R. GROSS, G. EHRENSVÄRD and L. GARNJOBST, 1954 Synthesis of aromatic compounds by Neurospora. Proc. Nat. Acad. Sci. 40:271-276.

TROLAND, L. T., 1917 Biological enigmas and the theory of enzyme action. Amer. Nat. 51:321-350.

UMBREIT, W. W., W. A. WOOD and I. C. GUNSALUS, 1946 The activity of pyridoxal phosphate in tryptophane formation by cell-free enzyme preparations. Jour. Biol. Chem. 165:731-732.

VILLEE, C. A., and H. B. BISSELL, 1948 Nucleic acids as growth factors in Drosophila. Jour. Biol. Chem. 172: 59-66.

VILLEE, C. A., and M. D. LOWENS, 1948 Barbituric acid as an inhibitor of growth and pupation in Drosophila. Genetics 33:632-633 (abstr.).

WADDINGTON, C. H., 1940 Organizers and Genes. Cambridge Univ. Press. London and New York. pp i-v + 1-160.

WAELSCH, H., 1952 "Certain aspects of intermediary metabolism of glutamine, asparagine, and glutathione", pp 237-319, in: Advances in Enzymology, Vol. XIII, ed., F. F. Nord. Interscience Publishers, Inc. New York.

WAGNER, R. P., 1944 The nutrition of D. mulleri and D. aldrichi. Growth of the larvae on cactus and the microorganisms found in cactus. Univ. Texas Publ. No. 4445: 104-128.

WAGNER, R. P., 1949 The in vitro synthesis of pantothenic acid by pantothenicless and wild type Neurospora. Proc. Nat. Acad. Sci. 35:185-189.

WAGNER, R. P., and H. K. MITCHELL, 1948 An enzymatic assay for studying the nutrition of Drosophila melanogaster. Arch. Biochem. 17:87-96.

WAGNER, R. P., and B. M. GUIRARD, 1948 A gene-controlled reaction in Neurospora involving the synthesis of pantothenic acid. Proc. Nat. Acad. Sci. 34:398-402.

WAGNER, R. P., and C. H. HADDOX, 1951 A further analysis of the pantothenicless mutants of Neurospora. Amer. Nat. 85:319-330.

WAGNER, R. P., and H. K. MITCHELL, 1955 Genetics and Metabolism. John Wiley and Sons, Inc. New York. Chapman and Hall, Ltd. London. pp i-xi + 1-444.

WATSON, J. D., and C. H. CRICK, 1953a A structure for deoxyribose nucleic acid. Nature 171:737-738.

WATSON, J. D., and C. H. CRICK, 1953b The structure of DNA. Cold Spring Harbor Symposia on Quant. Biol. 18:123-131.

WATSON, J. D., and C. H. CRICK, 1953c Genetical implications of the structure of deoxyribonucleic acid. Nature 171: 964-967.

WHELDALE, M., 1916 The Anthocyanin Pigments of Plants. Cambridge Univ. Press. London and New York. pp i-xi + 1-318.

WHITE, M. J. D., 1945 Animal Cytology and Evolution. Cambridge Univ. Press. London and New York. pp i-viii + 1-375.

WOLKOW, M., and E. BAUMANN, 1891 Über das Wesen der Alkaptonurie. Hoppe-Seyl. Ztschr. f. physiol. Chem. 15:228-285.

WOMACK, M., and W. C. ROSE, 1934 Feeding experiments with mixtures of highly purified amino acids. VI. The relation of phenylalanine and tyrosine to growth. Jour. Biol. Chem. 107:449-458.

WRIGHT, S., 1934 Physiological and evolutionary theories of dominance. Amer. Nat. 68:24-53.

WRIGHT, S., 1941 The physiology of the gene. Physiol. Rev. 21:487-527.

WRIGHT, S., 1942 The physiological genetics of coat color of the guinea pig. Biol. Symposia 6:337-355.

WRIGHT, S., 1945 Genes as physiological agents. Amer. Nat. 79:289-303.

YANOFSKY, C., 1952a The effects of gene change on tryptophan desmolase formation. Proc. Nat. Acad. Sci. 38:215-226.

YANOFSKY, C., 1952b Tryptophan desmolase of Neurospora. Partial purification and properties. Jour. Biol. Chem. 194:279-286.

APPENDIX A

Reprinted for private circulation from
PHYSIOLOGICAL ZOÖLOGY
Vol. XXIV, No. 4, October 1951
PRINTED IN U.S.A.

AMINO ACIDS AND GROWTH FACTORS IN A CHEMICALLY DEFINED MEDIUM FOR DROSOPHILA[1,2]

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As has been realized, a chemically defined medium and growth under aseptic conditions are prerequisites both for exacting studies of the nutrition of organisms and for determining the biochemical difference of genetically different strains. Such studies on multicellular animals have been few in comparison to those on other organisms. That *Drosophila* could be raised in the absence of microörganisms was demonstrated early in the century (Delcourt and Guyénot, 1911; Loeb and Northrop, 1915; and Baumberger, 1919), but a chemically defined medium that will support normal and rapid growth is only now being perfected. However, synthetic media which support growth (slow, but otherwise almost normal, development) of *Drosophila* have been in use in several laboratories (Schultz *et al.*, 1946, etc.; Begg and Robertson, 1948, etc.). Such advance has been made with few other multicellular animals. Much information is available concerning the nutritional requirements of *Aedes aegypti* (Trager, 1935, etc.), but whole casein is still used in the synthetic medium. There is still less information concerning the nutritional requirements of other multicellular animals. Therefore, a more comprehensive study of the medium used for *Drosophila* was suggested.

Some of the nutritional requirements for *Drosophila* were found by Guyénot (1917), Bacot and Harden (1922), van't Hoog (1935, 1936), and Tatum (1939). The first announcement of a chemically defined medium was by Schultz *et al.* (1946) in an abstract, but the quantities of ingredients used were not published. Villee and Bissell (1948) published a formula "essentially similar to Schultz'," using casein hydrolysate. They reported that only about 70 per cent of the larvae pupated and only about 40 per cent became adults, which is far below the normal development. Begg and Robertson (1948, 1950) and Begg (1949) presented a medium (containing casein and gelatin) and stated that it would permit complete, though slow, development. They presented no data to indicate the percentage of pupation or percentage to become adults.

In the light of this paucity of data in the literature regarding a chemically defined medium, it seems pertinent to report at this time the results of a series of studies in this laboratory which have led to a medium, chemically defined (except for agar), which permits growth more rapid than previously reported and which allows all or nearly all the larvae to pupate and become adults.

[1] The work was done under a Grant-in-Aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[2] We are extremely grateful to Doctors G. W. Kidder and Virginia Dewey for advice, suggestions, and criticisms throughout the course of this work. We wish to thank Mr. Richard Buffington for the work on tryptophane, and Mr. Stewart Price for the work on DL-isoleucine.

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MATERIAL AND METHODS

All studies in this series were made with a wild-type strain (Oregon-R) of *Drosophila melanogaster*. Eggs were collected on small flat dishes containing yeast and a corn meal–molasses agar medium from adults in a large glass jar. The eggs were washed in several baths of sterile Ringer's solution and several baths of 70 per cent alcohol, and, when free from all visible particles, they were placed in fresh 70 per cent alcohol. After 45 minutes they were inoculated by means of a flattened wire into sterile tubes of the synthetic medium, using standard bacteriological precautions.

The media used in the various sets of experiments differed (though consistent within a set of experiments), since any improvements discovered in one would be incorporated in the next. The various media are listed in Table 1, and are referred to in the text by their appropriate numbers.

In the preparation of the medium, the amino acids (except cystine) and sucrose were weighed in amounts designed for 100 ml. of final medium, ground together in a mortar, and dissolved in 50 ml. of distilled water, which included the amount of NaOH necessary to neutralize the solution. To this were added, from stock solutions, nucleic acid, the phosphates, the chlorides, the sulphates (except $FeSO_4$, which was made up each time), and the growth factors (except pteroyl glutamic acid, which was in 20 per cent alcohol). The stock solution of the growth factors was made anew each week. Next were added the cystine (dissolved in 4 ml. of 1 N HCl), $FeSO_4$, and pteroyl glutamic acid. The solution was again neutralized and the total volume brought up to 100 ml. with distilled water. Agar was then added and the mixture heated to the boiling point. The medium was then pipetted in 4-ml. amounts into test tubes containing cholesterol. Finally, the tubes were plugged with nonabsorbent cotton, autoclaved for 15 minutes at 15 pounds pressure, and slanted until solidified.

Ten eggs were inoculated per tube, and the tubes were kept at 25° C. (except where noted). Twenty-four hours after inoculation the number of larvae per tube was recorded. Larval counts were made again on the fifth day, and each subsequent day until pupation and eclosion were completed. The tubes were then tested for sterility by adding nutrient broth, incubating them for 48 hours at 37° C., and then streaking some of the contents onto sterile nutrient agar plates, which were then also incubated. Any tubes proving to have a contamination were not considered in the results.

The over-all picture of growth was measured by considering the percentage of the original number of larvae which pupated, the average time in days from egg to pupa, and the percentage of pupae to hatch into adults. Other factors were not considered, since the percentage of eggs to hatch is fairly constant regardless of the medium, as is the time spent in the pupa case. Measurements have been made of larval length and width and of adult weight, but the variation within a single tube was frequently as great as the average variation between experimental sets.

TRYPTOPHANE

Drosophila larvae will not survive without tryptophane (Lafon, 1939). To study this requirement further, dose responses to DL-tryptophane and L-tryptophane (the form of this substance found in nature) were run, with concentrations ranging from 0.003 mg/ml up to 15.0 mg/ml added to a medium which contained only 12 other amino acids (No. 1, Table 1). At the lower concentrations

TABLE 1

COMPOSITION OF VARIOUS SYNTHETIC MEDIA

Material	No. of Medium				
	1	2*	3	4	5
	Mg/Ml				
Alanine	.....	.....	L—1.085	S	.....
Arginine	L—0.794	S	S	S	S
Aspartic acid	.....	.....	L—1.221	S	.....
Cystine	L—0.400	L—0.480	S	S	S
Glutamic acid	L—4.418	S	S	S	S
Glycine	0.387	S	S	1.745	0.387
Histidine	L—0.484	S	S	S	S
Hydroxyproline	.....	.....	L—0.384	S	.....
Isoleucine	DL—1.258	S	L—1.260	S	DL—1.258
Leucine	L—2.345	S	S	S	S
Lysine	L—1.337	S	S	S	S
Methionine	DL—0.339	S	S	L—0.339	DL—0.339
Phenylalanine	L—1.008	S	S	S	S
Proline	.....	.....	L—1.682	S	.....
Threonine	DL—0.756	S	S	S	S
Tryptophane	L—0.349	S	S	L—1.745	L—0.349
Tyrosine	.....	.....	L—1.240	S	.....
Valine	DL—1.355	S	DL—1.355	L—1.355	DL—1.355
Sucrose	5.0	S	S	7.5	5.0
Cholesterol	0.1	S	S	S	S
Ergosterol	.....	.....	.....	1.0	.....
Lecithin	0.1	S	.....	.....	0.1
Ribonucleic acid	1.0	3.0	1.0	S	3.0
Inosine	.....	.....	.....	0.25	.....
Thymine	.....	.....	.....	0.004	.....
	μg/Ml				
Biotin	0.0094	S	0.020	S	0.0094
B_{12}	0.004	S	S	0.04	.....
Ca-pantothenate	6.0	S	S	S	S
Choline chloride	20.0	S	S	S	S
Pteroyl glutamic acid	6.0	S	S	S	S
Pyridoxine	30.0	S	3.0	S	S
Riboflavin	2.4	S	S	S	S
Thiamine	1.5	S	S	S	S
Protogen	2.5 units	S	.....	.....	.....
B_T	9.375	.....	.....	.....	9.375
Inositol	42.0	S	S	.....	42.0
p-Aminobenzoic acid	2.0	S	S	.....	2.0
Niacinamide	10.0	S	S	S	S
Constants†	.....	.....	.....	.....	.....

* "S" indicates that the amount is the same as that shown in the preceding column.

† All media contained the following:

Salts (Tatum, 1939):	Mg/Ml
$MnSO_4 \cdot 4H_2O$	0.246
$MgSO_4 \cdot 7H_2O$	0.0129
$FeSO_4$	0.0129
KH_2PO_4	0.606

Salts (Tatum, 1939):	Mg/Ml
K_2HPO_4	0.606
NaCl	0.0129
$CaCl_2$	0.0129
Agar	15.0

there was a distinct level below which there was no growth. With L-tryptophane, this point was in the vicinity of 0.06 mg/ml; with DL-tryptophane, about 0.15 mg/ml. Fair growth (pupation in 14 days) was achieved at concentrations of L-tryptophane slightly less than 0.1 mg/ml and continued to show some improvement up to about 1.5 mg/ml (pupation in about 11.5 days); with DL-tryptophane, fair growth was obtained at concentrations slightly less than 0.2 mg/ml, and very slight improvement up to about 6.0 mg/ml (12.5 days). With L-tryptophane there was no significant change in growth up to concentrations around 3.0 mg/ml, but in concentrations higher than that the dose-response curve began to fall (the length of time necessary for pupation increasing) until, at a concentration of 9.0 mg/ml, 22 days were required for pupation. Higher concentrations would not support growth. With DL-tryptophane there was no significant change in growth up to concentrations as high as 7.0 mg/ml. Concentrations from 9.2 to 15.0 mg/ml were slightly inhibitory (an inhibition was noted by Wilson [1945] with a concentration of tryptophane [DL?] of 4.08–8.16 mg/ml of a medium containing 5 per cent yeast). DL-tryptophane in concentrations above 15.0 mg/ml was not soluble in the medium. Schultz and Rudkin (1948) and Rudkin and Schultz (1949) worked with tryptophane in concentrations of from 0.06 to 0.23 mg/ml. However, they did not state which form of tryptophane was used or what was the effect upon growth except as it pertained to the niacinamide requirement or to the comparison of the requirements of mutant and wild type.

It is suggested by the dose-response data that *Drosophila* does not utilize the unnatural form of tryptophane, since, in general, about twice the amount of the racemic mixture is required to elicit the growth response obtained with the natural form.

A marked phenotypic change occurred at concentrations of L-tryptophane as high as 6 mg/ml. All the flies showed abnormalities of various types—for example, tumors throughout the body, deformed heads, wavy bristles, barlike eyes, abnormal tarsi, darker body color, and darkened eyes. The dark eye color was not brown, as Wilson (1945) found when, in addition to yeast, high concentrations of tryptophane were used in the medium. Rather, the eye appeared to contain the usual amount of red pigment which had darkened, as in the case of sepia and some other eye color mutations in *Drosophila*.

In the earlier phases of the work various ingredients were added to a 2 per cent killed yeast medium to test their possible toxicity. It was found then that tryptophane in high concentrations inhibited growth and that high concentrations of ribonucleic acid would overcome the inhibition (Hinton *et al.*, 1949). It was realized later that with yeast in the medium the results were extremely variable and frequently could not be repeated. For that reason those data are not presented and can be used only as a rough approximation of the true situation. Using the same (No. 1) chemically defined medium, an antagonistic action of nucleic acid to the inhibition exhibited by tryptophane was found to exist but not to be so striking. Data are presented in Table 2 to support the contention that the inhibition caused by tryptophane can, at least in part, be overcome by additional amounts of nucleic acid. The medium for the experiment was divided into four parts: Part 1 served as the control (0.349 mg/ml of L-tryptophane and 1 mg/ml of ribonucleic acid); part 2 contained the higher concentration of tryptophane (6 mg/ml); part 3 was the same as part 2, except that it contained, in addition, an

increased amount of nucleic acid (3 mg/ml); and part 4 was like the control except that it contained the increased amount of nucleic acid. As seen in Table 2, the average time of pupation was increased by 4 days when the amount of tryptophane was increased, but was increased by only 1.6 days when the high concentration of nucleic acid was also present.

TABLE 2

PARTIAL RELEASE OF A TRYPTOPHANE INHIBITION BY NUCLEIC ACID

Part	Original No. Larvae	Per Cent Larvae To Pupate	Average Time to Pupation (Days)	Per Cent Pupae To Become Adults
1	40	100	13.2	97
2	45	85	17.1	58
3	40	97	14.8	60
4	30	100	13.3	87

TABLE 3

DOSE RESPONSE TO DL-ISOLEUCINE AT 27°.5 C.
(12 Other Amino Acids in Medium)

Conc. (Mg/Ml)	Average Day of Pupation
0.00	Larvae died
0.05	Larvae died
0.10	Larvae died
0.20	Larvae died
0.40	21.2
1.25	9.6
4.00	12.6
6.25	13.1
8.00	14.1
16.00	16.0
40.00	20.1

ISOLEUCINE

Isoleucine was demonstrated to be a probable requirement for *Drosophila* by Lafon (1939). That it is a requirement is verified as indicated in Table 3 (medium No. 2, Table 1). The dose-response curve (as measured in this case by the average time required for pupation, in days) indicates a narrow range of tolerance by *Drosophila* to isoleucine. Above optimum concentration it became increasingly toxic.

As with tryptophane, there is an indication that the toxicity of isoleucine can be partially overcome with increased amounts of nucleic acid (medium No. 2). The experiment was divided into four parts (Table 4).

TABLE 4

DESIGN OF EXPERIMENT TO TEST THE RELEASE OF AN ISOLEUCINE INHIBITION BY NUCLEIC ACID

Part	Amount of Nucleic Acid (Mg/Ml)	Amount of DL-Isoleucine (Mg/Ml)
1	3.0	1.26
2	3.0	6.66
3	5.0	6.66
4	5.0	1.26

TABLE 5

PARTIAL RELEASE OF AN ISOLEUCINE INHIBITION BY NUCLEIC ACID AT VARIOUS TEMPERATURES

Temp. (° C.)	Degree of Inhibition by Isoleucine (Days)	Amount of Improvement with Increased Nucleic Acid (Days)
19	3.5	−0.3
20	2.8	+0.3
23	2.8	+0.8
25	3.6	+1.2
27.5	3.1	+0.6

An experiment was completed at each of five temperatures ranging from 19° to 27°.5 C. Shown in Table 5 are the degree of inhibition (measured by the average day of pupation) (part 2 compared to part 1) and the improvement in growth occasioned by the addition of increased nucleic acid (part 3 compared to part 2).

Although the differences are not great, they suggest that the antagonistic action of nucleic acid to the inhibition exhibited by isoleucine is affected by temperature. Nucleic acid is most efficient at 25° C., being less so at a higher temperature and becoming completely ineffective at the lowest temperature tested. Similar results were found to exist in another strain of *Drosophila* tested on a different medium (Hinton *et al.*, 1949).

A study of the ability of *Drosophila* to use just the natural isomer of isoleucine, though not extensive, indicates that growth is somewhat improved at the concentration used (1.26 mg/ml, which represents twice the amount of L-isoleucine expected in the same weight of the racemic mixture). The results of the comparison are given in Table 6 (medium No. 3, Table 1).

TABLE 6

COMPARISON OF GROWTH WITH L- AND DL-ISOLEUCINE

Isoleucine	Original No. Larvae	Per Cent Pupation	Av. Time To Pupation (Days)	Per Cent Pupae To Become Adults
DL....	26	92.3	11.5	83.3
L.....	34	97.1	11.1	87.8

GLYCINE

It was discovered that the correct concentration of glycine was of the utmost importance in obtaining good growth. Originally the amount used was selected as an approximation of that in casein (about 0.1 mg/ml of medium). Subsequently, a concentration of glycine of 0.3876 mg/ml was tried. Examination of Table 7 reveals clearly the marked improvement in growth when the concentration of glycine was approximately four times that found in casein (the medium used was like No. 1 in Table 1) except that no B_T was used). However, Table 7 is an average of a number of sets in which B_{12}, protogen, and the concentration of pyridoxine were experimental variables. Since comparable sets showed the same improvement, they are not presented individually.

A dose response to glycine at concentrations of 0 to 4.845 mg/ml of medium in the presence of 17 other amino acids showed the optimal amount to be about 1.8 mg/ml. Three such experiments were run and gave similar results (medium No. 3 for one; No. 4 for the others). Concentrations less than 0.388 mg/ml gave much slower growth, and concentrations above 2.326 mg/ml became increasingly toxic.

TABLE 7

COMPARISON OF TWO CONCENTRATIONS OF GLYCINE

Conc. of Glycine (Mg/Ml)	Original No. Larvae	Per Cent Pupation	Av. Time To Pupation (Days)	Per Cent Pupae To Become Adults
0.0969.....	181	74.5	17.5	67.4
0.3876.....	557	88.2	12.2	93.1

SERINE

Rudkin and Schultz (1949) reported D-serine as toxic and referred to "sublethal concentrations," thereby implying that it killed the organisms in high concentrations. Our work verifies this conclusion. Using 1.26 mg. of DL-serine (a little less than the amount of serine in casein) per milliliter of various media (No. 1; No. 2 plus B_T; No. 5; and each with different amounts of glycine), the growth of 122 larvae was studied. No pupae were formed. In another experiment, 150 larvae were studied (media Nos. 2 and 5, each with 18 other amino acids). Six days after inoculation of the eggs, only 57 larvae remained alive; on

the tenth day, only 1 was left, and it died without pupating. In the control set (without serine) there was 100 per cent pupation and 93 per cent eclosion. Of the 57 larvae still alive on the sixth day in the set with serine, 16 were in twelve tubes containing 1 mg/ml of ribonucleic acid, while the remaining 41 were in nine tubes containing 3 mg/ml of ribonucleic acid. This may indicate that nucleic acid (as in the case of tryptophane and isoleucine) has a slight detoxifying effect.

The effect of L-serine was studied next. At the time, to our knowledge, only one commercial concern offered the natural form. Using this in concentrations of 1.26 mg/ml, about 100 larvae were studied (medium No. 1). Approximately 50 per cent pupated, in an average of 16.3 days, and only 5 pupae became adults (control: about 80 per cent pupation, 12.3 days, and 70 per cent eclosion). This would indicate that either some D-serine or some other impurity injurious to *Drosophila* was included, since some L-serine, obtained later from a different source,[5] was only slightly toxic. The results, using the pure L-serine, are somewhat puzzling (Table 8). If the differences obtained are to be considered significant, they can be explained only by assuming that L-serine (in the amount used) is slightly inhibitory and that the inhibition can be overcome by increased amounts of nucleic acid in the presence of thirteen other amino acids, but not in the presence of eighteen.

[5] For the pure L-serine we are most grateful to Dr. J. Greenstein, of the National Institute of Public Health, Bethesda, Maryland.

TABLE 8

GROWTH WITH AND WITHOUT L-SERINE
(Medium No. 1 or No. 2 with Variables as Noted in Table)

No. Amino Acids in Medium (Besides Serine)	L-Serine (Mg/Ml)	Amount of Nucleic Acid (Mg/Ml)	No. Larvae	Per Cent Pupation	Av. Time to Pupation (Days)	Per Cent Adults
18	0.00	1.0	52	96.2	11.9	90.4
18	1.26	1.0	54	92.6	11.8	87.0
18	1.26	3.0	105	85.7	12.1	80.0
13	0.00	1.0	52	96.2	12.2	88.5
13	1.26	1.0	52	77.6	14.6	71.4
13	0.00	3.0	92	87.6	12.2	83.2
13	1.26	3.0	57	85.2	10.9	81.4

ARGININE AND THE "ORNITHINE CYCLE"

Arginine was reported as an essential amino acid for *Drosophila* by Lafon (1939); this is clearly verified by the present study (Table 9), as all larvae died without arginine on an otherwise complete medium. The concentration of arginine (0.794 mg/ml) used in these experiments is within the range found by Wilson (1944) to be tolerated by *Drosophila*.

Since the place of arginine in the ornithine cycle has been studied in detail from a chemicogenetic point of view in other organisms (Srb and Horowitz, 1944), it seemed of interest to study, in so far as possible, the relationships of arginine in *Drosophila* nutrition. Only a preliminary study has been completed, and it is presented in Table 9. From these data it is indicated that the arginine requirement can be met only in part by ci-

trulline and not at all by ornithine,[6] at least in the concentrations used. The presence or absence of aspartic acid in the medium appears to make no difference.

OTHER AMINO ACIDS

The results of a number of experiments indicate that growth is better when 18 of the amino acids are used than when only 13 are used. The 13 used were the 10 amino acids essential for the rat (and for *Drosophila* [Rudkin and Schultz, 1947]) plus glycine, cystine, and glutamic acid. The 18 used included these 13, plus alanine, aspartic acid, proline, hydroxyproline, and tyrosine. The natural forms were used in these experiments except that the racemic mixture was used in the case of isoleucine, methionine, valine, and threonine (row 2, Table 10). In more recent experiments all the natural forms were used with the one exception of DL-threonine (row 3, Table 10). Row 4, Table 10, shows the results when the natural form of methionine was used with the racemic mixture of the other 3 amino acids under consideration. Thus this set differs from row 2 only in the form of methionine used. Likewise, row 5, Table 10, differs from row 2 only in that the natural form of valine was used. Otherwise the media were as shown in No. 3, Table 1. The use of other natural forms compared to the use of the racemic mixture has been presented in preceding sections. In all cases there appears to be a very slight improvement in growth when the natural form is substituted for the racemic mixture. The improvement (except

TABLE 9

ATTEMPTS TO REPLACE ARGININE
(Medium No. 2, minus Lecithin)

Arginine (Mg/Ml)	Aspartic Acid (Mg/Ml)	Citrulline (Mg/Ml)	Ornithine (Mg/Ml)	No. Larvae	Per Cent Larvae To Pupate	Av. Time to Pupation (Days)	Per Cent Larvae To Become Adults
0.794	.....	.....	.....	42	83.3	12.2	83.3
.....	.....	.....	.....	45	0.0	.....	0.0
.....	1.221	0.794	.....	41	26.8	29.2	2.4
.....	.....	0.794	.....	53	17.0	27.1	3.8
.....	1.221	.....	.....	57	0.0	.....	0.0
.....	1.221	.....	0.794	57	0.0	.....	0.0

TABLE 10

13 AMINO ACIDS COMPARED TO 18 AMINO ACIDS
(Various Combinations of L- and DL-Forms: See Text)

Row	No. Amino Acids	No. Larvae	Per Cent Larvae To Pupate	Av. Time to Pupation (Days)	Per Cent Larvae To Become Adults
1.....	13	1,073	84.5	13.6	75.7
2.....	18	547	91.6	11.8	84.6
3.....	18	29	100.0	11.1	96.6
4.....	18	36	91.7	11.4	93.9
5.....	18	34	97.1	11.2	93.9

[6] We are grateful to Dr. Milton Heinrich, Amherst College, for supplying us with the citrulline and ornithine.

with serine) is not striking enough to warrant the conclusion that the racemic mixtures are highly toxic, as reported by Schultz *et al.* (1946).

Although marked improvement is obtained by using 18 amino acids, no one amino acid when added to the original 13 gave this improvement, and in all but one case growth was slower than when only the 13 were used, as measured in days to pupation (Table 11).

TABLE 11

NONESSENTIAL AMINO ACIDS
(Medium No. 2 plus B_T)

In Addition to 13 Amino Acids	Average Time to Pupation (Days)
None	11.9
L-alanine	12.5
L-aspartic acid	12.1
L-proline	13.1
L-hydroxyproline	12.9
L-tyrosine	11.7

BIOTIN

Schultz *et al.* (1946) found that biotin was one of the factors necessary for the growth of *Drosophila*, previously missing from the synthetic medium. In the present study a dose response to biotin was carried out by growing the larvae at each of 21 concentrations of biotin (Table 12) ranging from 0.0 to 500 mγ/ml (medium No. 2 plus B_T). In the absence of biotin in the synthetic medium, larval growth was retarded or stopped. Most larvae died with only about 10 per cent pupating (requiring about 20 days), and in no case were adults formed (from about 150 original larvae). Concentrations as low as 0.05 mγ/ml of medium had a definite effect upon the larval survival to the eleventh day, but the percentage of larvae which pupated was not increased, nor did any adults emerge. When the concentration was increased to 0.4 mγ/ml, the percentage of pupation was increased, and some adults appeared. The over-all picture of growth continued to improve as the concentration of biotin was increased, until a concentration of biotin of 16.7 mγ was reached. Thereafter, there was no appreciable improvement. No toxic effect of biotin was obtained, even in the presence of 500 mγ/ml. Trager (1948) suggested a possible toxic effect upon *Aedes* in the presence of 166.7 mγ/ml of biotin; Fraenkel and Blewett (1943) reported a toxic effect for *Tribolium* in the presence of 250 mγ/gm of diet. *Drosophila* also differs from these other insects in that the optimal concentration of biotin for it is lower (around 20 mγ/ml as opposed to nearly 50 for *Aedes* and 100 for *Tribolium*); and the minimal amount of biotin is lower for *Drosophila* (4.2 mγ/ml), an amount that did not allow the emergence of *Aedes* adults.

TABLE 12

DOSE RESPONSE OF *Drosophila* TO BIOTIN

Concentration of Biotin (Mγ/Ml)	No. Larvae	Per Cent Larvae To Pupate	Av. Time To Pupation (Days)	Per Cent Larvae To Become Adults
0	116	10.5	19.3	0.0
0.05	8	12.5	30.0	0.0
0.1	10	0.0	.....	0.0
0.4	9	77.8	20.7	11.1
0.5	8	75.0	22.7	0.0
0.8	8	75.0	19.5	25.0
0.9	7	71.4	19.6	14.0
4.2	25	84.0	17.6	40.0
5.0	8	87.5	17.6	37.5
6.7	32	84.4	14.7	62.5
8.0	7	100	16.0	85.7
10.0	27	74.0	15.5	55.6
13.3	34	82.4	14.2	64.7
16.7	63	93.1	12.3	82.8
33.3	39	89.7	13.3	79.5
66.7	45	93.3	11.5	84.0
136.0	45	95.6	11.6	91.1
200.0	39	97.4	11.8	87.2
275.0	41	90.2	12.2	75.6
350.0	54	90.7	11.4	87.0
425.0	58	94.8	11.7	81.0
500.0	60	93.3	11.9	83.3

Trager (1948), working with the mos-

quito (*A. aegypti*), investigated the relationship of biotin and fat-soluble biotin-active substances in the nutrition of that organism. He found lipid compounds could at least partially replace biotin, suggesting the importance of biotin in their synthesis. It seemed of interest to study such a relationship in another insect and in the absence of casein. Experiments similar to, but not identical with, those designed by Trager were carried out. Lecithin was chosen as the lipid to be used, as it was one of those tested by Trager and has been reported by Delcourt and Guyénot (1911) as having a stimulatory effect upon the development of *Drosophila*. The summarized results in Table 13 (medium No. 2 plus B_T, Table 1) show that, in the absence of biotin, lecithin even in amounts as high as 400,000 mγ/ml allowed no better growth than was obtained in the absence of both lecithin and biotin. A sparing action of lecithin could not be demonstrated, since growth was not improved with concentrations of lecithin as high as 400,000 mγ/ml in the presence of a minimal amount of biotin (4.2 mγ/ml). Nor could any stimulation of growth by lecithin be demonstrated, even with concentrations of lecithin as high as 400,000 mγ/ml in the presence of an optimal amount of biotin (16.7 mγ/ml). Since lecithin showed no activity, it can be concluded that the sample used was biotin-free.

Since the work of Koser *et al.* (1942), of Stokes *et al.* (1947), and of others has demonstrated an interrelation between biotin and aspartic acid and since biotin has been shown to play a role in the conversion of ornithine to citrulline (McLeod *et al.*, 1949), the possibility existed that the failure here to demonstrate the partial replacement of biotin by lecithin was due to the failure of one of these other biochemical cycles. In the light of this possibility, experiments were run to test the ability of lecithin to replace biotin when aspartic acid and citrulline were present in the medium (No. 2 plus B_T). The results (Table 14) fail to show that, in the nutrition of *Drosophila*, biotin is involved in the synthesis of lecithin. There is, however, an indication that citrulline can spare biotin, since, when citrulline was present in addition to a suboptimal amount of biotin, growth was significantly improved. The data are not

TABLE 13

EFFECT OF LECITHIN IN THE ABSENCE OF BIOTIN AND IN THE PRESENCE OF MINIMAL AND OPTIMAL AMOUNTS

Lecithin (Mγ/Ml)	No Biotin				Biotin (4.2 Mγ/Ml)				Biotin (16.7 Mγ/Ml)			
	No. Tubes*	No. Pupae	Av. Time	No. Adults	No. Tubes*	No. Pupae	Av. Time	No. Adults	No. Tubes*	No. Pupae	Av. Time	No. Adults
0	18	36	19.1	0	9	77	17.3	10	3	26	11.9	24
5,000	5	18	21.2	0	6	43	17.2	17	.....	.....	.....	.....
15,000	6	18	20.4	0	6	49	17.3	22	.....	.....	.....	.....
30,000	14	12	24.0	0	7	64	18.2	19	3	28	12.3	22
100,000	4	0	.....	0	3	29	18.6	18	.....	.....	.....	.....
120,000	2	0	.....	0	3	28	19.0	13	.....	.....	.....	.....
150,000	4	3	23.3	0	.....	.....	.....	.....	4	31	12.3	28
200,000	4	1	24.0	0	6	37	18.3	11	2	16	11.9	15
400,000	6	5	19.5	0	6	48	18.3	16	5	52	14.2	41

* Approximately 10 eggs per tube.

complete enough to attempt an explanation of the three adults (5.1 per cent of the original larvae) which eclosed in the absence of biotin when aspartic acid was in the medium (row 2, Table 14). These were the only adults to appear in any experiment where biotin was absent from the medium.

being six other variables involved in the total series of experiments. The results show that, regardless of the medium used, in each of the sixteen comparisons the lower concentration of pyridoxine allowed faster growth and the average time of pupation was less in every case (from 0.3 to 2.0 days faster—one case

TABLE 14

EFFECT OF LECITHIN IN THE ABSENCE OF BIOTIN AND IN THE PRESENCE OF MINIMAL AND OPTIMAL AMOUNTS AND WITH ASPARTIC ACID OR ASPARTIC ACID AND CITRULLINE IN THE MEDIUM

Lecithin (Mγ/Ml)	No Biotin				Biotin (4.2 Mγ/Ml)				Biotin (16.7 Mγ/Ml)			
	No. Larvae	No. Pupae	Av. Time	No. Adults	No. Larvae	No. Pupae	Av. Time	No. Adults	No. Larvae	No. Pupae	Av. Time	No. Adults
	Aspartic Acid (1.2 Mg/Ml)											
200,000	51	3	19.8	0	56	51	18.5	15	.....	.....	.....	.....
400,000	59	14	18.4	3	53	50	18.2	14	.....	.....	.....	.....
	Aspartic Acid (1.2 Mg/Ml) and Citrulline (1.2 Mg/Ml)											
0	29	1	21	0	23	21	15.4	11	41	40	12.2	37
30,000	36	1	24	0	45	40	15.0	27	40	38	13.0	34
100,000	36	6	18.2	0	38	37	14.9	23	45	41	13.1	36
200,000	42	5	19.9	0	39	36	14.6	24	40	37	12.7	28
400,000	40	6	15.0	0	41	38	15.5	25	46	43	13.0	39

PYRIDOXINE

In the light of the findings of Kidder and Dewey (1949*b*), working with *Tetrahymena*, that pyridoxine was necessary in relatively large amounts for optimum growth, experiments were carried out to test higher concentrations with *Drosophila*. In sixteen pairs of sets of tubes a comparison was made of the effect on growth of concentrations of pyridoxine of 3.0 and 30.0 μg/ml of medium. Each set of tubes within a pair of sets contained exactly the same medium except for the difference in the amount of pyridoxine. Any pair might differ, however, from other pairs as to the basal medium used, there

based on only 7 larvae was 3.3 days faster and is not included—with a total average for all sets of 1 day faster, based on a total of 1,080 larvae).

Pyridoxine has been reported as a requirement for *Drosophila* (Tatum, 1941). In the present work dose responses to pyridoxine have given inconsistent results. It is conceivable that some nucleic acids carry contaminations of the growth factor and this possibility is being tested.

VITAMIN B_{12}

When 0.004 mγ of vitamin B_{12} were added per milliliter of medium, there was a consistent, though small, improve-

ment in growth (Table 15). This is true in the ten pairs of sets of tubes available for comparison. An examination of the data reveals that the improvement in growth was probably due solely to a beneficial effect of B_{12} for pupation. (A pair is considered as two sets of tubes, identical except that one contains B_{12} and the other does not. The medium in one pair, however, may contain some factors not present in the medium of the other pairs.) When the average times of pupation (days after inoculation) in the sets without B_{12} are compared to the time in the sets with B_{12}, the two can be seen to be the same. They are always within $\pm$ 1 day of each other. The same is true if the percentage of pupae to become adults is considered. The variation is in both directions. However, when the same consideration was given to the percentage of larvae to pupate, it was found that a higher percentage pupated in each case in the set with B_{12} added (except in 1 case, where the difference was only 1.8 per cent). This higher percentage of pupation with B_{12} added is considered significant, since it was so consistent.

TABLE 15

COMPARISON OF GROWTH WITH AND WITHOUT VITAMIN B_{12} IN THE MEDIUM

With B_{12}				Without B_{12}			
No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Pupae To Become Adults	No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Pupae To Become Adults
25	100	16.9	68.0	13	76.9	15.9	60.0
26	92.3	18.1	58.3	17	82.4	19.1	14.3
24	95.8	16.6	52.2	10	80.0	18.5	37.5
19	84.2	16.9	56.3	37	78.6	17.6	40.9
34	100	16.9	47.1	38	94.7	16.2	47.1
35	94.3	18.0	45.5	30	90.0	18.2	55.6
63	88.9	12.4	96.4	51	80.4	13.6	85.4
62	99.7	16.4	93.2	29	86.2	12.0	94.3
26	92.5	18.1	97.9	53	94.3	12.0	94.0
34	100	16.9	71.4	48	83.3	16.1	80.0
Av.	94.8	16.7	68.6	Av.	84.7	15.9	60.9

PROTOGEN

Since protogen,[7] the growth factor so named by Stokstad *et al.* (1949), was found to be essential to *Tetrahymena* (Dewey, 1941, 1944; Kidder and Dewey, 1949*a*), it seemed advisable to see whether it was a factor required by *Drosophila*. The results of ten sets with protogen added (0.0025 units/ml) compared to a comparable set in each case lacking protogen (Table 16) indicated that protogen is not required by *Drosophila*, nor does the addition of it to the medium have any influence on growth, under the conditions of the experiment. It can be noted in Table 16 that only a small variation plus or minus was obtained when the percentage of larvae to pupate, the percentage of pupae to become adults, or the average time to pupation was considered. Since there was no consistency in these results and since the improvement shown by some nearly cancels the decrease shown by others, the foregoing conclusion was drawn.

The possibility remained that the agar

[7] We are grateful to Dr. G. W. Kidder, Amherst College, for supplying us with protogen.

furnished a source of this growth factor. This possibility was tested by comparing the growth of larvae raised on standard Bacto-Agar, as ordinarily used, and on dialyzed Bacto-Agar. Table 17 gives the results, which indicate no significant differences (medium No. 4, Table 1).

pairs (116 larvae), the set containing the B_T concentrate (9.9 γ/ml) was slower to pupate than was the control set (107 larvae) (0.5, 0.6, 1.1, 2.6, and 2.7 days slower, respectively). The other three sets (63 larvae) were faster than their controls (79 larvae), but only by 0.1, 0.3, and 0.3 days, respectively. This is interpreted to mean that B_T at the concentration used has a slight inhibitory effect on the rate of growth of *Drosophila*. However, as with B_{12}, the percentage of larvae to pupate was higher when the B_T concentrate was present in the medium, but the percentage of pupae to eclose as adults was not significantly different. The percentage of pupation with B_T was from 3.1 to 11.9 higher in seven of the

TABLE 16

COMPARISON OF GROWTH WITH AND WITHOUT PROTOGEN IN THE MEDIUM

With Protogen				Without Protogen			
No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Pupae To Become Adults	No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Pupae To Become Adults
37	78.6	17.6	40.9	17	82.4	19.1	14.3
38	94.7	16.2	47.1	10	80.0	18.5	37.5
28	92.9	16.4	46.2	25	100	16.9	68.0
19	84.2	16.9	56.3	26	92.5	18.1	58.3
34	100	16.9	47.1	24	95.8	16.6	52.2
35	94.3	18.0	45.5	18	100	17.8	50.0
63	88.9	12.4	96.4	75	87.0	12.9	93.1
35	74.3	17.4	65.4	48	83.3	16.1	80.0
88	91.1	12.2	92.5	53	94.3	12.0	94.0
51	80.4	13.6	85.4	29	86.2	12.0	94.3
Av.	87.9	15.8	62.3	Av.	90.2	16.0	64.2

TABLE 17

TEST FOR PRESENCE OF PROTOGEN IN AGAR

	No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Adults
No protogen, dialyzed agar	37	94.6	10.4	100
Progoten,* dialyzed agar	32	81.3	10.8	100
No protogen, standard agar	28	96.4	10.9	96.3
Protogen,* standard agar	23	87.0	10.6	100

* 0.0025 units/ml.

Fraenkel and Blewett (1947) found a factor necessary for the growth of the insect *Tenebrio* and called it "B_T." It was suggested that B_T might also be stimulatory for *Drosophila*, and it was therefore tried in the medium in eight sets of tubes, each set having a control set containing the same medium but minus B_T.[8] In five

[8] We are grateful to Dr. G. Fraenkel, of the University of Illinois, for furnishing the B_T concentrate and suggesting the concentration to be used.

cases (average increase was 7.8 per cent); a single case was less by 4.2 per cent. The percentage of pupae to become adults was higher in the set containing B_T in three cases and lower in four cases, indicating that B_T had no effect on eclosion. Despite the indication that the B_T concentrate stimulates pupation, its use is not being continued, since it seems to retard larval development and since B_{12} alone seems to stimulate pupation as much or more.

OTHER VITAMIN B REQUIREMENTS

Riboflavin is a definite requirement of *Drosophila* (van't Hoog 1935, 1936; Tatum, 1939). Without it in the medium, all larvae die (Table 18). Pteroyl glutamic acid (Schultz *et al.*, 1946) and Ca-pantothenate (Tatum, 1941) are necessary for rapid growth and pupation (Table 18); but most of the larvae do not die immediately in their absence, since they lived, in the case of Ca-pantothenate, for 29 days. When choline is omitted, growth proceeds at a normal rate, but no pupation occurs. Inositol seems to be unnecessary, no difference in growth resulting because of its presence or absence in the medium (Table 18). Since growth and development are improved when para-aminobenzoic acid is omitted, it can be concluded that this factor is not essential or stimulatory to *Drosophila*. It is assumed that Begg and Robertson realized this, since they do not list the factor in the most recent report of their medium (1950). Niacinamide (Tatum, 1939) and thiamine (van't Hoog, 1935, 1936) have each been reported as requirements for *Drosophila*. Our results agree with their findings (Table 18).

TABLE 18

SOME GROWTH FACTORS
(Medium No. 3 minus B_{12})

Omitted from Medium	No. Larvae	Per Cent Pupation	Av. Time to Pupation (Days)	Per Cent Pupae To Become Adults
None	39	94.9	10.5	100.0
Para-aminobenzoic acid	26	100.0	9.0	100.0
Ca-pantothenate	8	0.0	.....	0.0
Choline chloride	11	0.0	.....	0.0
Pteroyl glutamic acid	8	12.5	16.0	0.0
Inositol	30	96.7	10.3	100.0
Niacinamide	20	0.0	.....	0.0
Riboflavin	12	0.0	.....	0.0
Thiamine	37	0.0	.....	0.0

UNIDENTIFIED FACTORS

Tatum (1939) and Schultz *et al.* (1946) reported a still unidentified factor for rapid development, present in the water-insoluble fraction of yeast. Begg and Robertson (1950) found an alkali-soluble fraction necessary for completely normal development. Since the medium described here admittedly does not support such rapid growth as does yeast (Table 19), several yeast fractions[9] were added to the medium (Nos. 1 and 4, Table 1) to study the effect of a possible unidentified factor lacking in the medium. One yeast fraction (A) was the filter cake from

[9] We are grateful to the Difco Laboratories and to Dr. H. W. Schoenheim, director of the bacteriological laboratories, Difco Laboratories, for providing the yeast fractions and the descriptions of them.

the preparation of Bacto-Yeast Extract left in the press following the first extraction of the autolyzed yeast. Another sample (B) was the residue from the second extraction of the autolyzed yeast. Both residues were dried *in vacuo* and powdered. These were prepared by the Difco Laboratories in the same manner as those they furnished Tatum (1939).

It may be seen (Table 19) that improvement was achieved by the yeast fraction when 12 individual amino acids were used or when Stearns's "parenamine"[10] was used as the amino acid source. However, in the presence of 18 individual amino acids, development was somewhat accelerated with fraction A, which contains, in addition to the autolyzed yeast cells, a small amount of soluble compounds, but was not accelerated with fraction B, which contains a very small amount, if any, of the soluble compound. This was true on both media Nos. 1 and 4. It may indicate that whatever was supplied previously by such fractions is already present in our medium, which contains 18 amino acids (but lacking or in too low a concentration in "parenamine" and the 12 amino acid mixture), but is not entirely effective because of the imbalance of factors. This would suggest that, when the proper balance of the amino acids is achieved, the present medium may support rapid growth without the addition of unidentified factors.

TABLE 19

UNIDENTIFIED GROWTH FACTORS

No. Amino Acids in Medium	Yeast Fraction	Av. Time to Pupation (Days)
No. 1 12 (no glycine)	None	19.9
No. 1 12	B	13.5
No. 1 12	A	10.2
No. 1 18	None	14.9
No. 1 18	B	14.2
No. 1 18	A	10.5
Parenamine	None	13.4
Parenamine	B	8.8
No. 4 18	None	9.2
No. 4 18	B	9.7
None	Whole yeast	5.4

TABLE 20

GROWTH ON THE CHEMICALLY DEFINED MEDIUM NO. 4 GIVEN IN TABLE 1

Temp. (° C.)	No. Larvae	Per Cent Larvae To Pupate	First Day of Pupation	Last Day of Pupation	Av. Time to Pupation (Days)	Per Cent Pupae To Become Adults
25	43	97.7	7.0	9.5	7.5	97.6
28	42	85.7	5.5	8.5	6.7	97.2

THE MEDIUM

In the light of the results of all the foregoing experiments and the literature on the subject (Schultz *et al.*, 1946; Begg and Robertson, 1950; Hinton *et al.*, 1950), a medium has been derived which includes those factors shown to be necessary or stimulatory and in amounts as near optimal as possible. This medium is presented as No. 4 (Table 1), and the picture of growth resulting on this medium is presented in Table 20.

Actually, this medium is the sum total of all individual improvements found in the various experiments. No attempt has been made to alter each ingredient individually in the present mixture. It is

[10] A modified casein hydrolysate suggested by Dr. Jack Schultz (personal communication).

planned to perform such experiments, and until that time no claim can be made that all ingredients are necessary or in the proper concentration in the medium as it is now constituted. It is only too clearly indicated by the various experiments that the optimal concentration of one ingredient may vary, depending upon the balance of other factors.

DISCUSSION

The development of a chemically defined medium on which *Drosophila* can be raised in the absence of microörganisms has progressed to the point that exacting studies can now be made of the role of various amino acids and growth factors in the nutrition of this insect. This medium supports normal development, and most of the larvae become adults. However, larval development is prolonged for 1 or 2 days, indicating that either some growth-stimulating factor is absent from the medium or that the balance of nutrilites already in the medium is not adjusted for optimal development. The latter hypothesis is being tentatively adopted. It may be recalled (Table 19) that, with 18 amino acids present in the medium as the sole source of amino acids, no improvement in the rate of growth could be demonstrated by the addition of a a yeast fraction found by Tatum (1939) and Schultz *et al.* (1946) to contain an unidentified growth-stimulating factor. The unidentified growth factor continually fails to stimulate growth as the medium becomes progressively refined. Further work is in progress to attempt to adjust the balance, especially of the amino acids. This becomes a still more logical approach, since Begg and Robertson (1950) found an alkali-soluble yeast fraction which, when added to casein, gelatin, and tryptophane as the amino acid source, gave the normal rate of development and which shows nucleoprotein-like reaction.

During the course of the work on the amino acid balance, a relationship of nucleic acid to amino acids was noted. In the case of tryptophane, isoleucine, and serine (the only amino acids tested in this connection), high concentrations of the amino acid resulted in an inhibition of growth which was partially overcome by the addition of higher-than-usual concentrations of nucleic acid. With just nucleic acid in the higher concentration, the growth was usually somewhat inhibited. This relationship existed when only 12 other amino acids were present in the medium. At least in the case of serine, when 18 other amino acids were present, there was a negative effect with the addition of extra nucleic acid. Why such a relationship should exist is difficult to explain. It is conceivable, however, that, in the building of nucleoproteins by the organism, the various amino acids are in some way dependent upon the presence of nucleic acid.

That higher levels of glycine were found to be so important in bettering the growth is not surprising, since Kidder and Dewey (1945) found in *Tetrahymena* that glycine seems to function as a detoxifying agent for the essential amino acids. The high amounts of glycine used in the medium for *Drosophila* would also account for the lack of need for serine which was shown by these experiments (Kidder and Dewey, 1947).

Although more extensive studies are needed and are in progress to study the relation of arginine to other nutrilites in the nutrition of *Drosophila*, the preliminary results reported here indicate an inability to convert ornithine to citrulline. However, these results do show a relationship of biotin, citrulline, and arginine, since arginine may be partially replaced by citrulline and since biotin, in turn, is spared by citrulline.

These studies of the nutritional requirements of *Drosophila* do not reveal

any major deviations from the general nutritional pattern shared by all animals so far investigated. Minor differences are known to exist between the various animals studied (see Kidder and Dewey, 1947, for a comparison of *Tetrahymena* to mammals; and Albanese, 1947, for a review of the mammals). *Drosophila* is like some animals in certain respects and like others in some details. Thus *Drosophila*, like man, apparently cannot utilize the D-isomer of tryptophane, while *Tetrahymena* and the rat can. *Drosophila* differs from some animals, since it apparently does not complete the ornithine cycle and in this respect is more nearly like *Tetrahymena*. Insects differ from *Tetrahymena* in that they require biotin, but *Drosophila* differs from the other insects studied under comparable conditions in its quantitative requirements and its tolerance for biotin and the possible biotin-lecithin relationship. *Drosophila* differs from some microörganisms which are able to dispense with biotin in the presence of aspartic acid (Koser *et al.*, 1942; Stokes *et al.*, 1947) but is like some other organisms in that biotin seems to play a role in citrulline formation (McLeod *et al.*, 1949). Still other differences and similarities have been pointed out.

Of most general importance for optimal growth of *Drosophila* appears to be the balance of nutrilites, and this, undoubtedly, is a highly complex set of interrelationships, as was shown for *Tetrahymena* by Kidder and Dewey (1947) and suggested by Schultz and Rudkin (1949) and Rudkin and Schultz (1949) and by this study of some of the amino acids and growth factors in the nutrition of *Drosophila*.

SUMMARY

1. A series of experiments was made in an attempt to perfect a completely chemically defined medium for *Drosophila* that will support normal development.

2. The results indicated that *Drosophila* does not utilize D-tryptophane, metabolizing only the natural form. A syndrome of phenotypic abnormalities occurs when the flies are raised on high concentrations of L-tryptophane.

3. Tryptophane, isoleucine, and serine have an inhibitory effect on growth when present in high concentrations, and the data suggest that high levels of ribonucleic acid act as an antagonist to the inhibition, at least when only 13 amino acids are used in the medium.

4. Dose responses to tryptophane, isoleucine, glycine, biotin, and pyridoxine were carried out.

5. D-serine is extremely toxic to *Drosophila;* L-serine is slightly toxic under most circumstances.

6. The arginine requirement can be met in part by citrulline, but not by ornithine in the concentrations used. Biotin is spared by citrulline in the absence of arginine.

7. Glycine in high concentrations has a detoxifying effect on essential amino acids and, as a result, is most important for optimal growth.

8. Better results were obtained with 18 amino acids in the medium than when any other combination was used. The natural forms are generally best, although the racemic mixture gave growth almost as good in the case of some of the amino acids.

9. *Drosophila* tolerates concentrations of biotin over a large range, with no toxic effect demonstrable, as has been reported for some other insects. Nor could lecithin be shown to be able partially to replace biotin.

10. High concentrations of pyridoxine inhibited development. Vitamin B_{12} consistently raised slightly the percentage of larvae to pupate. Protogen could not be

shown to influence development or to be a requirement. B_T improved pupation very slightly but inhibited the rate of development to a small degree.

11. Other B-group vitamins were tested; inositol and para-aminobenzoic acid were found to be unnecessary and, in the case of para-aminobenzoic acid, to be somewhat inhibitory.

12. Various yeast fractions containing possible unidentified growth factors added to a complete medium failed to improve development.

13. A medium is presented which is completely chemically defined except for the agar base and which allows normal development of nearly 100 per cent of the eggs to the adult condition, with pupation occurring in 6–7 days (only 1–2 days slower than the normal). It is suggested that the normal rate of development can be achieved with further studies on the balance of nutrilites in the medium.

LITERATURE CITED

ALBANESE, A. A. 1947. The amino acid requirements of man. In: Advances in protein chemistry. **3**:227–64. New York: Academic Press.

BACOT, A. W., and HARDEN, A. 1922. Vitamin requirements of *Drosophila*. I. Vitamins B and C. Biochem. Jour., **16**:148–52.

BAUMBERGER, P. 1919. A nutritional study of insects, with special reference to microörganisms and their substrata. Jour. Exper. Zoöl., **28**:1–81.

BEGG, M. 1949. Nutritional requirements of *Drosophila*. Nature, **163**:881.

BEGG, M., and ROBERTSON, F. W. 1948. Nutritional requirements of *Drosophila*. Nature, **161**:769–70.

———. 1950. The nutritional requirements of *Drosophila melanogaster*. Jour. Exper. Biol., **26**:380–87.

DELCOURT, A., and GUYÉNOT, É. 1911. Génétique et milieu. Nécessité de la détermination des conditions. Sa possibilité chez les *Drosophiles*. Technique. Bull. sci. France et Belgique, **45**:249–333.

DEWEY, V. C. 1941. Nutrition of *Tetrahymena geleii* (Protozoa, Ciliata). Proc. Soc. Exper. Biol. and Med., **46**:482–84.

———. 1944. Biochemical factors in the maximal growth of *Tetrahymena*. Biol. Bull., **87**:107–20.

FRAENKEL, G., and BLEWETT, M. 1943. The vitamin B complex requirements of several insects. Biochem. Jour., **37**:686–92.

———. 1947. The importance of folic acid and unidentified members of the vitamin B complex in the nutrition of certain insects. *Ibid.*, **41**:469–75.

GUYÉNOT, É. 1917. Recherches expérimentales sur la vie aseptique et le développement d'un organisme (*Drosophila ampelophila*) en fonction du milieu. Thèse, Paris. 330 pp.

HINTON, TAYLOR, and ELLIS, JOHN. 1950. A nucleic acid requirement in *Drosophila* correlated with a position effect. Rec. Gen. Soc. Amer., **19**:104–5 (abstr.).

HINTON, TAYLOR; ELLIS, JOHN; and THERIAULT, D. L. 1949. Amino acid and nucleic acid relationships in *Drosophila*. Anat. Rec., **105**:33 (abstr.).

VAN'T HOOG, E. G. 1935. Aseptic culture of insects in vitamin research. Ztschr. f. Vitaminforsch, **4**:300.

———. 1936. Aseptic culture of insects in vitamin research. *Ibid.*, **5**:118.

KIDDER, G. W., and DEWEY, V. C. 1945. Studies on the biochemistry of *Tetrahymena*. I. Amino acid requirements. Arch. Biochem., **6**:425–32.

———. 1947. Studies on the biochemistry of *Tetrahymena*. X. Quantitative response to essential amino acids. Proc. Nat. Acad. Sci., **33**:347–56.

———. 1949*a*. Studies on the biochemistry of *Tetrahymena*. XI. Components of Factor II of known chemical nature. Arch. Biochem., **20**:433–43.

———. 1949*b*. Studies on the biochemistry of *Tetrahymena*. XII. Pyridoxine, pyridoxal and pyridozamine. *Ibid.*, **21**:58–65.

KOSER, S. A.; WRIGHT, M. H.; and DORFMAN, A. 1942. Aspartic acid as a partial substitute for the growth-stimulatory effect of biotin on *Torula cremoris*. Proc. Soc. Exper. Biol. and Med., **51**:204–5.

LAFON, M. 1939. Recherches sur quelques aspects du besoin qualitatif d'azote. Essai sur le besoin qualitatif d'azote chez un insecte: *D. melanogaster* Meig. Ann. d. physiol. and physiochim. biol., **15**:215–60.

LOEB, J., and NORTHROP, J. H. 1915. The salts required for the development of insects. Jour. Biol. Chem., **23**:431–34.

MCLEOD, P. R.; GRISOLIA, S.; COHEN, P. P.; and LARDY, H. A. 1949. Metabolic functions of biotin. III. The synthesis of citrulline to ornithine in liver tissue from normal and biotin deficient rats. Jour. Biol. Chem., **180**:1003–11.

RUDKIN, G. T., and SCHULTZ, JACK. 1947. Evolution of nutritional requirements in animals: amino-acids essential for *Drosophila melanogaster*. Anat. Rec., **99**:613 (abstr.).

———. 1949. A comparison of the tryptophane

requirements of mutant and wild type *Drosophila melanogaster*. Proc. 8th Internat. Cong. Genetics, pp. 652–53 (abstr.).

SCHULTZ, JACK, and RUDKIN, G. T. 1948. Absence of a sparing action of tryptophane on nicotinamide requirements of the fly, *Drosophila melanogaster*. Fed. Proc., **7**:185 (abstr.).

———. 1949. Nutritional requirements and the chemical genetics of *Drosophila melanogaster*. Proc. 8th Internat. Cong. Genetics, pp. 657–58.

SCHULTZ, JACK; ST. LAWRENCE, P.; and NEWMEYER, D. 1946. A chemically defined medium for the growth of *Drosophila melanogaster*. Anat. Rec., **96**:540 (abstr.).

SRB, A. M., and HOROWITZ, N. H. 1944. The ornithine cycle in *Neurospora* and its genetic control. Jour. Biol. Chem., **154**:129–39.

STOKES, J. L.; LARSEN, A.; and GUNNESS, M. 1947. Biotin and the synthesis of aspartic acid by microorganisms. Jour. Biol. Chem., **167**:613–14.

STOKSTAD, E. L. R.; HOFFMAN, C. E.; REGAN, M. A.; FORDHAM, DORIS; and JUKES, T. H. 1949. Observations on an unknown growth factor essential for *Tetrahymena gelcii*. Arch. Biochem., **20**:75–82.

TATUM, E. L. 1939. Nutritional requirements of *Drosophila melanogaster*. Proc. Nat. Acad. Sci., **25**:490–97.

———. 1941. Vitamin B requirements of *Drosophila melanogaster*. *Ibid.*, **27**:193–97.

TRAGER, W. 1935. On the nutritional requirements of mosquito larvae (*Aedes aegypti*). Amer. Jour. Hyg., **22**:475–93.

———. 1947. Insect nutrition. Biol. Rev., Cambridge Phil. Soc., **22**:148–77.

———. 1948. Biotin and fat-soluble materials with biotin activity in the nutrition of mosquito larvae. Jour. Biol. Chem., **176**:1211–23.

VILLEE, C. A., and BISSELL, H. B. 1948. Nucleic acids as growth factors in *Drosophila*. Jour. Biol. Chem., **172**:59–66.

WILSON, L. P. 1945. Tolerance of larvae of *Drosophila* for amino acids: tryptophane. Growth, **9**:145–54.

WILSON, L. P., and BIRCH, M. M. 1944. Tolerance of larvae of *Drosophila* for amino acids: arginine. Growth, **8**:125–48.

APPENDIX B

Reprinted from the Proceedings of the NATIONAL ACADEMY OF SCIENCES,
Vol. 37, No. 5, pp. 293–299. May, 1951

*AN ADENINE REQUIREMENT IN A STRAIN OF DROSOPHILA**

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Communicated by A. F. Blakeslee, March 1, 1951

With the improved growth now obtainable, the raising of *Drosophila melanogaster* upon a chemically defined medium under aseptic conditions,[1] renders possible more exacting studies of the nutritional requirements of genetically different strains. Thus a comparative study was made of a wild type (Oregon-R) and Inversion (2LR) 40d. The latter strain has been studied in detail in other respects[2, 3] and was found to possess a phenotype consisting of disarranged eye facets, mottled eye pigment, and

tumor-like black protrusions from the surface of the eye.[2] This phenotype was found to be directly correlated with the position of heterochromatin involved in a chromosomal inversion.[3] Furthermore, it was found that the abnormal phenotype could be caused to revert to the wild type, or normal, condition both when the position of the heterochromatin was changed by further chromosomal rearrangements[3] and by feeding the developing larvae an abundance of yeast at certain temperatures.[2] It appeared, therefore, that some factor in the food was needed by the inversion strain which was not required by the wild type in order to develop a normal eye. Since heterochromatin is also correlated with the development of the eye in the inversion strain, and since heterochromatin differs from euchromatin primarily in its nucleic acid synthesis,[4] it was suggested[2, 3] that the nucleic acid requirements of the two strains may differ. The present study was undertaken to test that hypothesis. Preliminary results were published earlier.[7]

Technique.—Eggs were collected and washed in 70% alcohol for 45 minutes. They were then inoculated, ten per tube, using bacteriological procedures, onto the surface of an agar slant of previously autoclaved medium. The base medium is given in table 4, and is a modification of the medium announced by Schultz, *et al.*[1] However, it should be noted that this medium is not in current use, a better medium having been devised.[5]

Growth was measured by an index derived by adding together the percentage of original larvae to pupate and the percentage of original larvae to become adults, and dividing that sum by the average time in days required for pupation. The larger the final figure so derived, the better the growth since the best growth would be in the case of a large percentage of pupation and eclosion in a short interval of time.

Each experimental set presented in the accompanying tables (except table 2) represents at least two independent experiments the results of which are added together.

Nucleic Acid Omitted from the Diet.—When the wild type strain was raised on medium containing no nucleic acid, the growth index was high (table 1), indicating, as has been reported by others,[1] that Drosophila does not require nucleic acid, normally being capable of synthesizing it. It may be noted, however, that growth is improved and the time required for pupation shortened upon the addition of nucleic acid to the medium.

On the other hand, the inversion strain proved to be incapable of completing development when nucleic acid was omitted from the diet (table 1). This clearly indicates that this strain, unlike the normal Drosophila, is incapable of synthesizing nucleic acid or some derivative of nucleic acid.

The Nucleic Acid Derivative Required.—Having established that the inversion strain failed to develop when nucleic acid was omitted from the diet, it remained to determine the nature of this requirement. The pre-

TABLE 1

		NO. LARVAE TO HATCH	PER CENT LARVAE TO PUPATE	AV. TIME TO PUPATION IN DAYS	PER CENT LARVAE TO BECOME ADULTS	GROWTH INDEX
Base medium plus nucleic acid (3 mg. per ml.)	wild type	31	100	16.2	74.0	10.7
	inversion	11	90.9	19.3	72.7	8.5
Base medium (no nucleic acid)	wild type	92	82.6	20.1	63.0	7.2
	inversion	59	6.8	25.2	0.0	0.3

vious experiment does not show whether the whole nucleic acid molecule is required by the organism, or only one or more of its constituents. Therefore, larvae were raised upon media lacking nucleic acid, but containing, in each case, one purine or pyrimidine base, ribonucleoside, or ribonucleotide. All were tried with the exception of cytosine which was unavailable at that time. Wild type larvae were used in half of the tubes in each experiment to serve as controls, except in the case of uridine where all the tubes became contaminated and could not be counted. The results are presented in table 2.

Inspection of table 2 shows that the inversion strain gave a good growth index only when adenine (or its nucleoside or nucleotide) was present in

TABLE 2

BASE MEDIUM PLUS		NO. OF LARVAE	PER CENT LARVAE TO PUPATE	AV. TIME TO PUPATION DAYS	PER CENT LARVAE TO BECOME ADULTS	GROWTH INDEX
Guanine (0.11 mg. per ml.)	wild type (+)	14	86	20.1	86	8.6
	inversion	28	32	22.3	21	1.3
Guanosine (0.165 mg. per ml.)	+	19	58	22.7	53	4.9
	inv.	23	0	..	0	0.0
Guanylic acid (0.25 mg. per ml.)	+	59	85	20.8	56	6.8
	inv.	11	9	21.0	9	0.9
Adenine (0.11 mg. per ml.)	+	12	83	20.5	83	8.1
	inv.	20	60	17.1	55	6.7
Adenosine (0.165 mg. per ml.)	+	39	97	18.5	95	10.4
	inv.	20	60	15.7	60	7.6
Adenylic acid (0.25 mg. per ml.)	+	15	80	19.3	53	6.9
	inv.	14	71	10.8	57	11.8
Uracil (0.11 mg. per ml.)	+	46	65	21.2	43	5.1
	inv.	37	0	..	0	0.0
Uridine (0.165 mg. per ml.)	+	..	..	..	..	..
	inv.	7	0	..	0	0.0
Uridylic acid (0.25 mg. per ml.)	+	39	74	23.1	67	6.1
	inv.	14	0	..	0	0.0
Cytidine (0.165 mg. per ml.)	+	32	81	22.1	75	7.1
	inv.	11	0	..	0	0.0
Cytidylic acid (0.25 mg. per ml.)	+	34	68	23.0	56	5.4
	inv.	56	0	..	0	0.0

the medium. In all other cases the larvae failed to develop except with guanine (9 pupae and 6 adults) and with guanylic acid (1 pupa and 1 adult). Rather than suppose guanine is replacing adenine, it is more logical to assume that the guanine and guanylic acid used in these experiments have a slight contamination of adenine. This is especially logical since guanosine does not support growth, and it is known to be more pure while guanylic acid is known to contain, at least, pyrimidine contaminants.[6]

TABLE 3

BASE MEDIUM PLUS BASES (0.11 MG. OF EACH, PER ML.)		NO. OF LARVAE	PER CENT LARVAE TO PUPATE	AV. TIME TO PUPATION, IN DAYS	PER CENT LARVAE TO BECOME ADULTS	GROWTH INDEX
Guanine, Adenine, Uracil	wild type (+)	29	86.2	17.7	55.2	8.0
	inversion	12	50.0	17.7	25.0	4.2
Guanine, Uracil	+	17	94.1	19.0	47.0	7.4
	inv.	10	0.0	..	0.0	0.0
PLUS RIBONUCLEOSIDES (0.165 MG. OF EACH, PER ML.)						
Guanosine, Adenosine, Uridine, Cytidine	+	48	85.4	15.3	54.2	9.1
	inv.	27	37.0	15.5	22.2	3.8
Guanosine, Uridine, Cytidine	+	34	64.7	22.5	20.6	3.8
	inv.	38	0.0	..	0.0	0.0
PLUS NUCLEOTIDES (0.25 MG. OF EACH, PER ML.)						
Guanylic acid, Adenylic acid, Uridylic acid, Cytidylic acid	+	33	90.9	15.9	42.4	8.4
	inv.	59	61.0	13.5	45.8	7.9
Guanylic acid, Uridylic acid, Cytidylic acid	+	38	65.8	20.6	26.3	4.5
	inv.	74	5.4	21.0	5.4	0.5

The wild type larvae gave fair growth indices (table 2) in all cases, although slight improvement was shown when adenine was present in some form and when guanine (with the supposed adenine contamination) was present. This leaves little doubt that the inversion strain differs in its nutritional pattern from the wild type in that it has lost the ability to synthesize adenine.

Combinations of Purines and Pyrimidines.—It remained to determine whether the adenine requirement of the inversion strain could be met by

any combination of purines and pyrimidines. Larvae were raised on media containing the purine and pyrimidine bases in all possible combinations; on media containing the purine and pyrimidine nucleosides in all possible combinations; and on media containing the purine and pyrimidine nucleotides in all possible combinations. The results of some of these are presented in table 3.

Table 3 can be summarized by pointing out that of all of the combinations of purines and pyrimidines tested, whether bases, nucleosides or nucleotides, none supported growth unless adenine (or adenosine, or adenylic acid) was in the combination. In no case where adenine (or

TABLE 4

BASE MEDIUM

	MG. PER ML.		MG. PER ML.
L-arginine	0.794	Cholesterol	0.1
L-cystine	0.48	Lecithin	0.1
L-glutamic acid	4.418	Sucrose	5.0
Glycine	0.388	KH_2PO_4	0.606
L-histidine	0.484	K_2HPO_4	0.606
DL-isoleucine	1.258	$CaCl_2$	0.0129
L-leucine	2.345	NaCl	0.0129
L-lysine	1.337	$FeSO_4 \cdot 7H_2O$	0.0129
DL-methionine	0.339	$MgSO_4 \cdot 7H_2O$	0.246
L-phenylalanine	1.008	$MnSO_4 \cdot 4H_2O$	0.129
DL-threonine	0.756	Agar	15.0
L-tryptophane	0.349		
DL-valine	1.355		
	GAMMA PER ML.		GAMMA PER ML.
Biotin	0.0094	Pteroylglutamic acid	6.0
Choline chloride	20.0	Pyridoxine	30.0
p-Aminobenzoic acid	2.0	Riboflavin	2.4
Inositol	42.0	Thiamine	1.5
Niacinamide	10.0	Vitamin B_{12}	0.004
Ca-pantothenate	6.0	Protogen	2.5 units[8]

adenosine, or adenylic acid) was omitted from the combination was a growth index higher than 0.6 obtained. In fact, in only two cases were any pupae obtained, and then only a total of 5. Since the results were consistent, data for the other combinations are not presented as they add nothing further to the conclusion.

It, therefore, is justifiable to conclude that the adenine requirement of the inversion strain cannot be satisfied by any combination of other purines and pyrimidines tested.

Discussion.—Since this is the first clear-cut case in animals of the inheritance of a basic biochemical difference involving a nutritional deficiency, it seemed fitting to publish the results at this time, even though the mode of inheritance cannot be conclusively stated.

As was pointed out at the beginning of the paper, it was the early studies on the behavior of the position effect known as In(2LR)40d which led to the initial attempts to search for differences in the nucleic acid requirement of this strain as compared to a wild type. That a difference was found does not prove that the difference (the adenine requirement) and the position effect are correlated, although the coincidence is highly suggestive. It can only be said, at this time, that in all crosses made the adenine requirement follows the chromosomal inversion in segregation, i.e., appears to be genetically linked to it. However, the techniques are complicated for distinguishing between the possibilities that the requirement is due to a gene associated with the inversion, and that it is an effect of the inversion itself. Nevertheless, a series of crosses to replace each chromosome is in progress, as well as a study of the requirements of reversions and partial reversions of the chromosomal inversion,[3] in an attempt to decide between these possibilities.

If the adenine requirement is due to the rearrangement of heterochromatin as a result of the inversion, it should then, in turn, be correlated with the phenotype manifested by the inversion. In the present work, phenotypic differences have been noted when the nucleic acid constituents of the medium were altered qualitatively or quantitatively. However, the natural variability of expression of this phenotype is great under most circumstances, and a true evaluation of the expression can be gained only by a statistical measurement of the mean degree of expression.[2] The present data are not extensive enough to make such a measurement valid. Also, the higher concentrations of nucleic acid in the diet become increasingly toxic thereby introducing the complicating factor of retarded growth. Any evaluation of the normal or abnormal development of the eye would have to correct for this factor. Experiments are in progress in an effort to clarify the nature of the correlation between adenine in the diet and the expression of the phenotype.

It seems certain that an adenine requirement is not a characteristic of all position effects. We have tested several different types of position effects without discovering another requirer. On the other hand, another adenine requirer has turned up in a strain which is thought to be cytologically normal.

When the strain requiring adenine is crossed with some other strains, the requirement usually behaves in the offspring as a dominant effect. However, when crossed to the Oregon-R wild strain, the requirement behaves as a recessive in the offspring. A more detailed analysis of this is in progress. It may be more than coincidental that an unexpected effect on the phenotype was found in earlier work[2] when the inversion strain was crossed to the Oregon strain.

The requirement for adenine can be overcome or partially so by altering

the type of medium used. Apparently several ingredients manifest an influence upon the requirement. The role of these various factors is under study. Because of their influence, it was necessary to use the present medium in order to demonstrate an "all-or-none" requirement, even though media supporting more nearly optimal growth are available.[5]

Even though the genetic analysis wants further elucidation, the fact that a multicellular animal has been found with a purine requirement offers the first opportunity for a detailed analysis of purine synthesis in the higher animals under chemically controlled conditions and in the absence of micro-organisms. Such a study is in progress.

* The work was done under a Grant-in-Aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. The research was aided by a Grant-in-Aid from the Sigma Xi Research Fund.

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[1] Schultz, Jack, St. Lawrence, P., and Newmeyer, D., *Anat. Rec.*, **96**, 540 (1946) (Abstr.).

[2] Hinton, Taylor, *Am. Nat.*, **83**, 69–94 (1949).

[3] Hinton, Taylor, *Genetics*, **35**, 188–205 (1950).

[4] Schultz, Jack, Caspersson, T., and Aquilonius, L., PROC. NATL. ACAD. SCI., **26**, 515–523 (1940).

[5] Hinton, Taylor, Noyes, D. T., and Ellis, John, *Physiol. Zool.* (in press).

[6] Kidder, G. W., and Dewey, V. C. (unpublished).

[7] Hinton, Taylor, and Ellis, John, *Rec. Gen. Soc. Am.*, **19**, 104–105 (1950) (Abstr.).

[8] Kidder, G. W., and Dewey, V. C., *Arch. Biochem.*, **20**, 433–443 (1949).

APPENDIX C

NOTE: See text-tables for identification of experiment.

Nine tube-rows contain fractions. The numerator of the first fraction in the row indicates the number of pupae (or adults) present in the tube on the day indicated at the head of the column. The denominator indicates the number of pupae (or adults) present in the tube on the day following that indicated at the head of the column. The fractions that follow continue in a similar manner.

Tube numbers followed by an asterisk (*) indicate that the results obtained were obviously heterogeneous for that tube. The figures given for the tubes so marked are not included in the total figures for the experiment.

Tube No.	Initial larval Inoc.	Numbers of Pupae: Number of days following inoculation										Total Pupae	Numbers of Adults: No. of days following inoculation							Total Adults
		4	5	6	7	8	9	10	11	12	13		7	8	9	10	11	12	13	
1	30	5	6	6	2	1	0	0				6	4	5	6	6				6
2	"	6	7	8	6	6	3	2				10	3	3	7	8				8
3	"	4	7	8	5	3	1	1				9	4	6	8	8				8
4	"	5	6	6	3	3	0	0				6	3	3	6	6				6
5	"	3	7	10	11	6	6	5				12	1	6	6	7				7
6	"	5	7	8	4	2	2	1				8	4	6	6	7				7
7	"	6	7	8	6	6	3	2				10	4	4	7	8				8
8	"	6	10	12	7	5	2	0				12	5	7	10	12				12
9	"	8	13	13	5	2	0	0				13	8	11	13	13				13
10	"	4	5	8	8	5	4	4				9	1	4	5	5				5
Total	300	52	75	87	57	39	21	15				95	31	55	74	80				80
11	30	-	3	7	15	19	21	19	14	2	0	23	-	-	2	4	9	21	23	23
12	"	-	4	12	13	14	12	13	8	6	0	20	-	-	3	7	12	14	20	20
13	"	-	5	8	12	15	16	15	11	0	0	22	-	-	3	7	11	22	22	22
14	"	-	1	10	13	13	14	16	12	2	0	18	-	-	1	2	6	16	18	18
15	"	-	3	8	12	16	19	19	13	2	0	22	-	-	1	3	9	20	22	22

Tube No.	Initial Larval Inoc.	Numbers of Pupae — Number of days following inoculation												Total Pupae	Numbers of Adults — No. of days following inoculation								Total Adults
		5	6	7	8	9	10	11	12	13	14	15	16		9	10	11	12	13	14	15	16	
16	30	2	9	14	19	22	20	14	4	1				25	3	5	11	21	24				24
17	"	-	6	10	12	14	20	10	1	0				22	-	2	12	21	22				22
18	"	3	8	14	16	17	16	13	2	0				22	1	4	12	22	22				22
19	"	4	12	14	16	17	16	13	2	0				18	1	2	5	16	18				18
Total	270	25	80	117	138	156	156	105	19	1				192	15	36	87	173	191				191
20	50	-	10	15	23	32	36	27	21	12	4	3		39	-	3	12	18	27	35	36		36
21	"	-	13	13	15	16	16	12	8	8	5	4		17	-	1	5	9	9	12	13		13
22	"	-	5	9	15	17	18	16	8	7	5	5		22	-	4	6	14	15	17	17		17
23	"	-	14	18	20	26	26	19	17	17	15	14		27	-	1	8	10	10	12	13		13
Total	200	-	42	55	73	91	96	74	54	44	29	26		105	-	9	31	51	61	76	79		79
24	50	-	3	8	16	23	27	30	21	14	10	8	7	37	-	3	7	16	23	27	29	30	30
25	"	-	7	15	19	25	32	26	20	12	7	4	1	38	-	5	12	18	26	31	34	37	37
26	"	-	11	11	16	21	20	15	5	5	4	4	4	28	-	8	13	23	23	24	24	24	24
27	"	-	5	14	25	31	33	29	23	17	11	7	4	37	-	2	7	14	20	26	30	33	33
Total	200	-	26	48	76	100	112	100	69	48	32	23	16	140	-	18	39	71	92	108	117	124	124
28	50	-	6	15	17	19	20	16	10	5	3	2	2	24	-	3	8	14	19	21	22	22	22
29	"	-	3	14	28	38	38	27	22	17	15	13	11	44	-	2	17	22	27	29	31	33	33

Tube No.	Initial Larval Inoc.	Numbers of Pupae: Number of days following inoculation												Total Pupae	Numbers of Adults: No. of days following inoculation								Total Adults
		6	7	8	9	10	11	12	13	14	15	16	17		10	11	12	13	14	15	16	17	
30	50	6	14	23	28	29	18	11	7	6	5	5		30	1	12	19	23	24	25	25		25
31	"	3	10	25	31	35	30	22	20	14	11	8		38	2	8	16	18	24	27	30		30
Total	200	18	53	93	116	122	91	65	49	38	31	26		136	8	45	71	87	98	105	110		110
32	50	10	19	19	30	30	21	16	11	7	4	4		35	5	14	19	24	28	31	31		31
33	"	12	23	23	30	29	23	14	9	7	7	6		37	7	14	23	28	30	30	31		31
34	"	13	23	26	31	31	24	22	14	8	7	5		36	4	12	14	22	28	29	31		31
35	"	6	15	30	34	32	23	20	9	5	5	4		38	3	14	18	29	33	33	34		34
Total	200	41	80	98	125	122	91	72	43	27	23	19		146	19	54	74	103	119	123	127		127
36	50	3	11	18	26	30	32	29	22	15	11	10	8	41	4	8	12	19	26	30	31	33	33
37	"	1	4	10	13	17	19	15	8	5	4	1	1	22	1	3	7	14	17	18	21	21	21
38	"	3	7	10	13	14	13	9	7	3	2	1	1	17	2	4	8	10	14	15	16	16	16
Total	150	7	22	38	52	61	64	53	37	23	17	12	10	80	7	15	27	43	57	63	68	70	70
39	50	-	3	17	28	31	35	33	28	20	12	11		41	-	2	8	13	21	29	30		30
40	"	1	8	18	25	35	36	33	24	17	10	8		47	3	7	14	23	30	37	39		39
41	"	-	12	17	24	25	17	14	8	8	6	6		32	1	13	18	24	24	26	26		26
Total	150	1	23	52	77	91	88	80	60	45	28	25		120	4	22	40	60	75	92	95		95
42	50	-	2	4	23	26	26	22	19	15	5	1		31	-	3	9	12	16	26	30		30

Tube No.	Initial Larval Inoc.	Numbers of Pupae: Number of days following inoculation 6	7	8	9	10	11	12	13	14	15	16	17	Total Pupae	Numbers of Adults: No. of days following inoculation 9	10	11	12	13	14	15	16	17	Total Adults
43	50	2	7	11	22	31	29	26	15	8	1	1		36	-	1	7	10	21	28	35	35		35
44	"	-	2	4	14	20	26	23	20	9	6	0		27	-	-	1	4	7	18	21	27		27
45	"	-	3	5	21	24	28	27	12	4	4	2		34	-	-	1	7	22	30	30	32		32
Total	200	2	14	24	80	101	109	98	66	36	16	4		128	-	1	12	30	62	92	112	124		124
46	50	1	5	6	28	31	18	15	8	2	0	0		32	1	1	14	17	24	30	32	32		32
47	"	1	3	7	11	17	19	14	6	5	4	4		22	-	1	3	8	16	17	18	18		18
48	"	-	4	8	11	13	18	17	8	6	1	0		22	-	1	2	5	14	16	21	22		22
49	"	2	6	17	20	17	9	7	7	6	6	6		25	2	7	16	18	18	19	19	19		19
Total	200	4	18	38	70	78	64	53	29	19	11	10		101	3	10	35	48	72	82	90	91		91
50	50	4	7	11	21	20	16	11	5	0	0	0		28	2	7	12	17	23	28	28	28		28
51	"	-	3	7	21	26	20	15	11	5	1	0		37	-	3	12	22	26	32	36	37		37
52	"	4	7	23	28	21	12	6	3	2	0	0		33	3	12	21	27	30	31	33	33		33
53*	"	2	2	3	4	4	4	2	2	2	2	2		5	1	1	1	3	3	3	3	3		3
Total	150	8	17	41	70	67	48	32	19	7	1	0		98	5	22	45	66	79	91	97	98		98
54	50	3	8	10	19	29	28	24	12	7	4	1	0	36	-	1	8	12	24	29	32	35	36	36
55	"	2	5	12	17	21	22	13	7	6	4	2	2	26	-	2	4	13	19	20	22	24	24	24
56	"	4	7	14	16	17	20	18	12	9	5	4	4	30	-	2	8	12	18	21	25	26	26	26

Tube No.	Initial Larval Inoc.	Numbers of Pupae: Number of days following inoculation												Total Pupae	Numbers of Adults: No. of days following inoculation									Total Adults
		6	7	8	9	10	11	12	13	14	15	16	17		9	10	11	12	13	14	15	16	17	
Total	150	9	20	36	52	67	70	55	31	22	13	7	6	92	-	5	20	37	61	70	79	85	86	86
57	50	4	10	11	15	19	14	11	9	2	1	1	1	23	-	3	9	12	14	21	22	22	22	22
58	"	3	10	12	15	21	20	13	9	7	2	1	0	27	-	2	7	14	18	20	25	26	27	27
59	"	2	3	6	7	14	15	13	6	2	1	1	0	18	-	1	2	5	12	16	17	17	18	18
Total	150	9	23	29	37	54	49	37	24	11	4	3	1	68	-	6	18	31	44	57	64	65	67	67
60	50	1	4	7	15	15	19	11	7	4	0			25	1	3	6	14	18	21	25			25
61	"	3	18	21	25	26	25	21	10	2	1			39	2	12	14	18	29	37	38			38
62	"	2	12	18	30	17	14	10	3	1	1			31	1	14	17	21	28	30	30			30
Total	150	6	34	46	70	58	58	42	20	7	2			95	4	29	37	53	75	88	93			93
63	25	1	1	6	8	10	8	5	3	0				13	-	1	5	8	10	13				13
64	"	2	6	7	10	9	6	4	3	0				10	-	1	4	6	7	10				10
Total	50	3	7	13	18	19	14	9	6	0				23	-	2	9	14	17	23				23
65	50	1	3	11	13	17	15	8	2	2	0	0		20	-	3	5	12	18	18	20	20		20
66	"	-	4	7	17	24	26	17	11	4	2	1		30	-	2	4	13	19	26	28	29		29
67	"	-	6	15	18	21	20	14	12	6	4	2		26	-	3	6	12	14	20	22	24		24
68	"	1	9	10	14	13	11	8	5	4	1	0		19	-	6	8	11	14	15	18	19		19
Total	200	2	22	43	62	75	72	47	30	16	7	3		95	-	14	23	48	65	79	88	92		92

Tube No.	Initial Larval Inoc.	Numbers of Pupae — Number of days following inoculation											Total Pupae	Numbers of Adults — No. of days following inoculation								Total Adults
		6	7	8	9	10	11	12	13	14	15	16		9	10	11	12	13	14	15	16	
69	50	-	3	14	22	26	20	15	11	8	2	0	27	-	1	7	12	16	19	25	27	27
70	"	1	3	12	14	17	14	10	5	1	0	0	19	-	2	5	9	14	18	19	19	19
71	"	1	2	7	18	22	19	17	7	3	2	1	23	-	1	4	6	16	20	21	22	22
72	"	1	5	10	17	17	15	11	8	3	0	0	18	-	1	3	7	10	15	18	18	18
Total	200	3	13	43	71	82	68	53	31	15	4	1	87	-	5	19	34	56	72	83	86	86
73	30	2	3	4	7	9	11	6	1	1			13	1	1	2	7	12	12			12
74	"	1	2	2	3	5	4	3	1	0			7	1	1	3	4	6	7			7
75	"	-	-	3	5	10	8	4	3	0			10	-	-	2	6	7	10			10
76	"	-	2	2	6	10	7	4	2	0			10	-	-	3	6	8	10			10
77	"	-	-	1	2	11	9	7	6	1			12	-	-	3	5	6	11			11
78*	"	-	-	-	1	4	6	4	4	1	0		6	-	-	-	2	2	5	6		6
79	"	-	3	5	6	7	7	6	3	0			10	-	2	3	4	7	10			10
Total	180	3	10	17	29	52	46	30	16	2			62	2	4	16	32	46	60			60
80	30	1	3	12	14	20	17	15	8	4	3		21	-	1	4	6	13	17	18		18
81	"	1	7	11	15	17	13	11	6	2	2		18	-	1	5	7	12	16	16		16
82	"	2	5	14	17	22	21	17	15	10	4		24	-	1	3	7	9	14	20		20
83	"	3	8	16	20	22	20	14	11	8	4		26	-	2	6	12	15	18	22		22

Tube No.	Initial Larval Inoc.	Numbers of Pupae — Number of days following inoculation												Total Pupae	Numbers of Adults — No. of days following inoculation									Total Adults
		6	7	8	9	10	11	12	13	14	15	16	17		9	10	11	12	13	14	15	16	17	
84	30	1	4	7	19	21	19	17	5	2	1			22	-	1	3	5	17	20	21			21
85	"	-	4	10	16	17	12	10	9	7	5			19	-	2	7	9	10	12	14			14
86	"	1	2	9	13	16	16	12	8	2	2			19	-	1	3	7	11	17	17			17
87	"	3	7	10	14	20	16	9	4	3	1			21	-	1	5	12	17	18	20			20
Total	240	12	40	89	128	155	134	105	66	38	22			170	-	10	36	65	104	132	148			148
88	10	2	2	4	5	5	5	4	4	3	3	1	0	6	-	1	1	2	2	3	3	5	6	6
89	"	1	1	2	2	2	3	3	3	2	1	1	0	4	1	1	1	1	1	2	3	3	4	4
Total	20	3	3	6	7	7	8	7	7	5	4	2	0	10	1	2	2	3	3	5	6	8	10	10
90	10	-	1	1	2	6	5	3	3	2	1	0	0	6	-	-	1	3	3	4	5	6	6	6
91	"	-	-	2	4	5	5	5	3	3	3	1	0	6	-	-	-	1	3	3	3	5	6	6
92	"	-	2	3	5	6	5	4	3	3	1	0	0	8	-	-	1	2	4	5	7	8	8	8
93	"	-	-	1	1	3	3	3	1	1	0	0	0	6	-	-	-	3	5	5	6	6	6	6
94*	"	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	0
Total	40	-	3	7	12	20	18	15	10	9	5	1	0	26	-	-	2	9	15	17	21	25	26	26
95	50	-	4	10	18	24	36	36	23	12	1	1		37	-	-	1	1	14	25	36	36		36
96	"	-	3	8	16	29	29	24	14	3	0	0		31	-	-	2	7	17	28	31	31		31
97	"	-	3	6	13	24	25	15	9	5	1	0		26	-	-	1	11	17	21	25	26		26

Tube No.	Initial Larval Inoc.	Numbers of Pupae: Number of days following inoculation 7	8	9	10	11	12	13	14	15	16	17	18	Total Pupae	Numbers of Adults: No. of days following inoculation 11	12	13	14	15	16	17	18	Total Adults
98	50	5	12	18	31	30	23	20	8	1	0			32	2	9	12	24	31	32			32
Total	200	15	36	65	108	120	98	66	28	3	1			126	6	28	60	98	123	125			125
99	30	1	1	3	5	10	12	10	6	3	2	1	1	14	1	2	4	8	11	12	13	13	13
100	"	-	1	1	3	5	9	11	8	5	5	3	2	12	-	-	1	4	7	7	9	10	10
101	"	-	2	4	7	12	15	14	12	8	6	4	3	17	-	1	3	5	9	11	13	14	14
102	"	-	1	3	6	10	14	13	11	7	6	4	2	18	-	2	5	7	11	12	14	16	16
103	"	1	2	2	5	11	15	16	14	10	7	4	4	17	-	1	1	3	7	10	13	13	13
104	"	1	3	4	5	7	9	9	7	5	3	1	1	11	1	2	2	4	6	8	10	10	10
105	"	-	1	1	4	4	6	7	6	4	3	1	0	9	-	1	2	3	5	6	8	9	9
106	"	-	2	3	6	8	12	13	10	7	6	3	3	15	-	1	2	5	8	9	12	12	12
Total	240	3	13	21	41	67	92	93	74	49	38	21	16	113	2	10	20	39	64	75	92	97	97
110	30	1	1	4	18	22	20	19	15	10	8	2	1	23	1	3	4	8	13	15	21	22	22
111	"	-	-	3	8	14	20	17	12	10	6	5	5	22	-	1	5	10	12	16	17	17	17
112	"	-	1	3	9	18	18	17	15	11	7	5	2	20	-	2	3	5	9	13	15	18	18
113	"	-	2	6	8	13	14	11	10	8	5	0	0	15	-	1	4	5	7	10	15	15	15
114	"	-	1	5	10	16	20	18	15	10	6	4	3	22	-	2	4	7	12	16	18	19	19
115	"	-	-	2	7	12	17	21	16	12	11	7	5	22	-	-	1	6	10	11	15	17	17

Tube No.	Initial larval Ince.	Numbers of Pupae — Number of days following inoculation												Total Pupae	Numbers of Adults — No. of days following inoculation									Total Adults
		7	8	9	10	11	12	13	14	15	16	17	18		10	11	12	13	14	15	16	17	18	
116	30	-	1	5	9	17	22	18	13	7	4	1	1	24	-	-	2	6	11	17	20	23	23	23
117	"	-	2	3	6	11	12	11	9	6	4	2	1	13	-	-	1	2	4	7	9	11	12	12
Total	240	1	8	31	75	123	143	132	105	74	51	26	18	161	-	1	12	29	56	87	110	135	143	143
118	30	2	3	5	6	6	7	5	3	3	1	0		10	-	1	3	5	7	7	9	10		10
119	"	-	4	4	5	5	4	4	3	3	3	2		5	-	-	1	1	2	2	2	3		3
120	"	1	2	4	7	8	6	6	3	1	1	1		9	-	1	2	2	5	8	8	8		8
121	"	2	3	7	9	10	7	4	4	3	1	1		11	-	1	4	7	7	8	10	10		10
122	"	-	1	3	5	5	4	3	1	1	0	0		5	-	-	1	2	4	4	5	5		5
123	"	2	5	11	12	16	15	12	11	7	6	5		19	-	2	4	7	8	12	13	14		14
Total	180	7	18	34	44	50	43	34	25	18	12	9		59	-	5	15	24	33	41	47	50		50
124	50	4	5	9	21	22	18	12	9	3	1	1	1	30	2	7	12	18	21	27	29	29	29	29
125	"	-	6	14	22	30	27	22	16	13	6	3	2	35	-	1	5	12	19	22	29	32	33	33
126	"	-	4	15	21	27	23	18	9	9	5	3	3	33	-	2	7	14	24	24	28	30	30	30
Total	150	4	15	38	64	79	68	52	34	25	12	7	6	98	2	10	24	44	64	73	86	91	92	92
127	50	-	7	13	13	17	19	9	7	3	2	1		20	-	-	1	11	13	17	18	19		19
128	"	5	17	24	26	16	2	2	1	0	0	0		28	-	11	26	26	27	28	28	28		28
129*	"	-	3	5	6	7	3	3	1	0	0	0		8	-	-	1	5	5	7	8	8		8

Tube No.	Initial Larval Inoc.	Numbers of Pupae — Number of days following inoculation											Total Pupae	Numbers of Adults — No. of days following inoculation							Total Adults
		7	8	9	10	11	12	13	14	15	16	17		11	12	13	14	15	16	17	
130*	50	1	1	3	4	6	4	2	0	0	0	0	6	-	2	4	6	6	6	6	6
Total	100	5	24	37	39	33	21	11	8	3	2	1	48	11	27	37	40	45	46	47	47
131	50	-	7	12	14	14	14	11	4	0			18	-	2	7	14	18			18
132	"	5	25	36	40	33	13	9	0	0			44	11	31	35	44	44			44
133	"	-	7	13	13	16	7	3	3	0			17	-	10	14	14	17			17
Total	150	5	39	61	67	63	34	23	7	0			79	11	43	56	72	79			79
134	50	-	3	12	15	16	10	5	4	1	1		17	-	7	12	13	16	16		16
135	"	1	16	18	24	18	12	3	2	2	1		34	15	22	31	32	32	33		33
136	"	1	15	21	22	13	5	1	1	0	0		25	12	20	24	24	25	25		25
Total	150	2	34	51	61	47	27	9	7	3	2		76	27	49	67	69	73	74		74
137*	50	-	1	6	7	7	6	3	2	0	0		7	-	1	4	5	7	7		7
138	"	5	9	16	20	17	13	11	0	0	0		20	3	7	9	20	20	20		20
139	"	1	8	20	23	22	17	8	7	0	0		28	6	11	20	21	28	28		28
140	"	2	6	8	10	11	19	13	11	3	1		25	3	6	12	14	22	24		24
Total	150	8	23	44	53	50	49	32	18	3	1		73	12	24	41	55	70	72		72
141	50	-	3	10	20	28	26	20	7	5	0		28	-	2	8	21	23	28		28
142	"	4	17	23	28	18	4	1	0	0	0		31	12	27	30	31	31	31		31

Tube No.	Initial Larval Inoc.	Numbers of Pupae: Number of days following inoculation											Total Pupae	Numbers of Adults: No. of days following inoculation							Total Adults
		7	8	9	10	11	12	13	14	15	16	17		11	12	13	14	15	16	17	
143	50	8	22	30	31	20	7	6	0	0	0		32	12	25	26	32	32	32		32
Total	150	12	42	63	79	66	37	27	7	5	0		91	24	54	64	84	86	91		91
144	50	8	24	26	30	23	15	6	2	1	0		33	10	18	27	31	32	33		33
145	"	-	5	11	20	22	19	16	14	6	0		23	-	4	7	9	17	23		23
146	"	6	23	39	42	29	9	2	1	1	1		43	14	34	41	42	42	42		42
147	"	3	15	28	34	24	7	4	2	0	0		34	10	27	30	32	34	34		34
Total	200	17	67	104	126	98	50	28	19	8	1		133	34	83	105	114	125	132		132
148	50	-	-	-	2	5	6	8	5	3	3	3	10	-	-	2	5	7	7	7	7
149	"	-	-	-	-	1	3	3	8	1	1	1	9	-	-	-	1	8	8	8	8
150	"	1	1	6	8	11	8	7	3	1	1	1	11	-	3	4	8	10	10	10	10
151	"	3	9	12	13	14	13	12	9	9	9	9	14	-	1	2	5	5	5	5	5
Total	200	4	10	18	23	31	30	30	25	14	14	14	44	-	4	8	19	30	30	30	30
152	50	1	7	17	23	35	35	26	16	12	10	8	40	1	4	14	24	28	30	32	32
153	"	-	8	16	24	31	35	22	17	5	2	1	38	-	3	16	21	33	36	37	37
154	"	1	7	12	18	21	26	23	15	13	9	7	31	-	4	8	16	18	22	24	24
155	"	2	3	7	13	18	20	20	11	5	2	2	22	1	2	2	11	17	20	20	20
Total	200	4	25	52	78	105	116	91	59	35	23	18	131	2	13	40	72	96	108	113	113

Tube No.	Initial Larval Inoc.	Numbers of Pupae: Number of days following inoculation											Total Pupae	Numbers of Adults: No. of days following inoculation								Total Adults
		8	9	10	11	12	13	14	15	16	17	18		11	12	13	14	15	16	17	18	
156	50	-	2	4	7	9	10	10	7	7	7	7	11	-	-	1	1	4	4	4	4	4
157	"	2	2	4	6	6	8	7	7	6	6	4	10	-	1	1	3	3	4	4	6	6
158	"	1	1	1	1	1	2	4	5	5	5	5	6	-	1	1	1	1	1	1	1	1
159	"	-	1	1	4	7	6	6	6	4	4	2	7	-	-	1	1	1	3	3	5	5
160	"	1	2	3	5	4	4	2	2	0	0	0	5	-	1	1	3	3	5	5	5	5
Total	250	4	8	13	23	27	30	29	28	23	23	18	39	-	2	5	9	12	17	17	21	21
161*	50	-	-	2	2	3	3	2	1	1	0		3	-	-	-	1	2	2	3		3
162	"	3	5	6	6	4	3	3	1	1	0		8	-	4	5	5	7	7	8		8
163	"	-	5	11	15	15	15	10	5	2	1		16	-	-	1	6	11	14	15		15
164	"	4	18	18	22	22	12	5	0	0	0		23	-	1	11	18	23	23	23		23
Total	150	7	28	35	43	41	30	18	6	3	1		47	-	5	17	29	41	44	46		46
165	50	7	9	13	20	26	13	4	3	3	3		21	1	5	8	12	17	18	18		18
166	"	3	5	11	19	22	23	16	6	4	2		30	1	4	7	14	24	26	28		28
Total	100	10	14	24	39	38	36	20	9	7	5		51	2	9	15	26	41	44	46		46
167	50	1	4	7	10	20	26	23	20	5	2	2	32	-	1	3	9	12	27	30	30	30
168	"	-	4	7	9	12	12	19	12	10	6	4	24	-	-	2	5	12	14	18	20	20
Total	100	1	8	14	19	32	38	42	32	15	8	6	56	-	1	5	14	24	41	48	50	50

Tube No.	Initial Larval Inoc.	Numbers of Pupae — Number of days following inoculation														Total Pupae	Numbers of Adults — No. of days following inoculation								Total Adults
		8	9	10	11	12	13	14	15	16	17	18	19	20	21		12	13	14	15	16	17	18	19	
169	50	-	2	6	18	32	30	19	10	2	0					38	-	8	19	28	36	38			38
170	"	3	7	11	30	30	25	16	10	6	1					31	1	6	15	21	25	30			30
171	"	4	21	34	38	31	24	20	6	0	0					40	9	16	20	34	40	40			40
Total	150	7	30	51	86	93	79	55	26	8	1					109	10	30	54	83	101	108			108
172	25	-	-	1	1	1	1	2	1	1	1	1	0			2	-	-	-	1	1	1	1	2	2
173	"	-	-	2	3	3	3	3	1	1	1	1	0			4	-	-	1	3	3	3	3	4	4
174	"	-	1	1	3	1	1	1	0	0	0	0	0			4	3	3	3	4	4	4	4	4	4
175	"	-	-	-	1	1	1	1	1	0	0	0	0			1	-	-	-	-	1	1	1	1	1
176	"	-	-	1	1	2	1	1	1	1	1	1	1			2	-	1	1	1	1	1	1	1	1
Total	125	-	1	5	9	8	7	8	4	3	3	3	1			13	3	4	5	9	10	10	10	12	12
177	25	-	1	9	10	15	17	10	2	1	0	0	0			18	-	1	8	16	17	18			18
178	"	-	-	2	2	5	9	15	12	10	8	$\frac{7}{5}$	$\frac{2}{0}$			15	-	-	-	3	5	7	$\frac{8}{10}$	$\frac{13}{15}$	15
179	"	-	-	5	9	13	16	11	9	7	4	$\frac{4}{1}$	$\frac{0}{0}$			16	-	-	5	7	9	12	$\frac{12}{15}$	$\frac{16}{16}$	16
180	"	-	-	3	7	11	14	14	13	11	6	3	3	1	1	14	-	-	-	1	3	8	$\frac{11}{11}$	$\frac{13}{13}$	13
181	"	-	-	4	11	14	16	16	15	12	12	7	4	2	0	16	-	-	-	1	4	4	$\frac{9}{12}$	$\frac{14}{16}$	16

Tube No.	Initial Larval Inoc.	Numbers of Pupae: Number of days following inoculation													Total Pupae	Numbers of Adults: No. of days following inoculation								Total Adults
		9	10	11	12	13	14	15	16	17	18	19	20	21		13	14	15	16	17	18	19	20	
Total	125	1	23	39	58	72	66	51	41	30	21	13	5	1	79	1	13	28	28	49	58/66	74/78		78
182	50	2	5	13	15	14	8	7	2						15	1	7	8	13					13
183	"	-	3	8	9	16	14	9	4						16	-	2	7	12					12
184	"	-	5	6	13	12	9	9	7						13	1	4	4	6					6
Total	150	2	13	27	37	42	31	25	13						44	2	13	19	31					31
185	50	1	12	14	26	31	25	14	9	4	3	3			32	1	7	18	23	28	29	29		29
186	"	3	7	11	21	30	26	20	16	9	2	1			30	-	4	10	14	21	28	29		29
187	"	-	2	5	12	19	22	17	10	4	4	1			22	-	-	5	12	18	18	21		21
Total	150	4	21	30	59	80	73	51	35	17	9	5			84	1	11	33	49	67	75	79		79
188	30	1	3	7	7	8	8	7	5	3	2	1	1		9	1	1	2	4	6	7	8	8	8
189	"	-	2	5	6	6	5	5	3	1	0	0	0		6	-	1	1	3	5	6	6	6	6
190	"	1	2	5	8	9	7	4	3	3	2	1	0		10	1	3	6	7	7	8	9	10	10
191	"	-	1	4	4	6	7	6	5	4	2	1	1		7	-	-	1	2	3	5	6	6	6
192	"	-	2	3	5	6	5	4	2	2	0	0	0		6	-	1	2	4	4	6	6	6	6
193	"	1	2	3	4	6	5	5	4	3	1	0	0		7	1	2	2	3	4	6	7	7	7
194	"	-	2	5	5	6	7	6	4	4	3	1	0		8	-	1	2	4	4	5	7	8	8

Tube No.	Initial Larval Inoc.	Numbers of Pupae: Number of days following inoculation												Total Pupae	Numbers of Adults: No. of days following inoculation								Total Adults
		9	10	11	12	13	14	15	16	17	18	19	20		13	14	15	16	17	18	19	20	
195	30	1	1	3	4	4	3	3	2	1	0	0	0	5	1	2	2	3	4	5	5	5	5
Total	240	4	15	35	43	51	47	40	28	21	10	4	2	58	4	11	18	29	37	48	54	56	56
196	30	2	3	5	7	12	9	7	4	1	0	0		13	1	4	6	9	12	13	13		13
197	"	-	-	2	3	5	5	4	2	1	0	0		5	-	-	1	3	4	5	5		5
198	"	-	-	1	4	4	5	4	4	4	3	3		5	-	-	1	1	1	2	2		2
199	"	-	1	3	4	7	7	6	3	1	0	0		8	-	1	2	5	7	8	8		8
200	"	-	-	1	1	2	3	4	2	2	1	0		4	-	-	-	2	2	3	4		4
201	"	-	1	1	3	5	4	4	4	2	1	1		5	-	1	1	1	3	4	4		4
202	"	1	1	3	7	6	6	3	2	2	1	0		8	1	2	5	6	6	7	8		8
203	"	-	1	2	2	5	5	4	4	1	0	0		6	-	1	2	2	5	6	6		6
Total	240	3	7	18	31	46	44	36	25	14	6	4		54	2	9	18	29	40	48	50		50
204	50	1	4	5	6	8	7	7	7	6	4	4		10	1	3	3	3	4	6	6		6
205	"	1	7	8	8	9	10	8	8	8	7	6		12	1	1	4	4	4	5	6		6
206	"	-	1	7	7	7	5	5	4	4	3	2		7	-	2	2	3	3	4	5		5
Total	150	2	12	20	21	24	22	20	19	18	14	12		29	2	6	9	10	11	15	17		17
210	25	-	-	-	1	3	3	2	2	2	2	2		3	-	-	1	1	1	1	1		1
211	"	-	-	-	2	2	2	0	0	0	0	0		2	-	-	2	2	2	2	2		2

Tube No.	Initial Larval Instar	Numbers of Pupae: Number of days following inoculation												Total Pupae	Numbers of Adults: No. of days following inoculation									Total Adults
		10	11	12	13	14	15	16	17	18	19	20	21		13	14	15	16	17	18	19	20	21	
212	25	1	3	5	6	7	5	4	4	2	1			8	-	1	3	4	4	6	7			7
213	"	1	1	4	5	6	6	4	4	1	1			7	-	1	1	3	3	6	6			6
214	"	-	1	1	2	2	1	0	0	0	0			2	-	-	1	2	2	2	2			2
Total	125	2	5	13	18	20	14	10	10	5	4			22	-	2	8	12	12	17	18			18
215	25	1	3	6	7	5	2	1	1					8	1	3	6	7	7					7
216	"	2	4	4	4	3	1	0	0					4	-	1	3	4	4					4
217	"	-	-	1	1	1	1	0	0					1	-	-	-	1	1					1
218	"	-	1	3	4	4	2	2	1					5	-	1	3	4	4					4
Total	100	3	8	14	16	13	6	3	2					18	1	5	12	15	16					16
219	30	-	1	7	12	14	20	16	15	9	4	2		21	-	-	1	5	6	12	17	19		19
220	"	-	-	3	6	10	16	15	12	6	4	1		17	-	-	1	2	5	11	13	16		16
221	"	-	-	2	7	9	12	10	7	6	2	0		14	-	-	-	4	7	8	12	14		14
222	"	-	1	6	8	16	17	14	10	9	3	2		19	-	-	2	5	9	10	16	17		17
223	"	-	1	5	7	12	14	13	8	5	3	2		16	-	-	2	3	8	11	13	14		14
Total	150	-	3	23	40	61	79	68	52	35	16	7		87	-	-	6	19	35	52	71	80		80
224	25	-	-	2	3	5	7	9	9	7	7	$\frac{5}{4}$	$\frac{4}{3}$	11	-	-	-	2	2	4	4	$\frac{6}{7}$	$\frac{7}{8}$	8

Tube No.	Initial Larval Inoc.	Numbers of Pupae — Number of days following inoculation											Total Pupae	Numbers of Adults — No. of days following inoculation							Total Adults
		11	12	13	14	15	16	17	18	19	20	21		15	16	17	18	19	20	21	
225	25	3	5	8	12	17	14	11	7	2	$\frac{2}{2}$	$\frac{2}{2}$	17	-	3	6	10	15	$\frac{15}{15}$	$\frac{15}{15}$	15
226	"	1	6	8	10	14	14	10	7	4	$\frac{4}{4}$	$\frac{2}{2}$	14	-	-	4	7	10	$\frac{10}{10}$	$\frac{12}{12}$	12
227*	"	-	-	-	1	1	1	2	2	2	$\frac{2}{1}$	$\frac{1}{1}$	2	-	-	-	-	-	$\frac{-}{1}$	$\frac{1}{1}$	1
228	"	4	7	12	16	16	15	12	8	3	$\frac{2}{2}$	$\frac{1}{1}$	16	-	1	3	8	13	$\frac{14}{14}$	$\frac{15}{15}$	15
Total	100	8	20	31	43	54	52	42	29	16	$\frac{13}{12}$	$\frac{9}{8}$	58	-	6	15	29	42	$\frac{45}{46}$	$\frac{49}{50}$	50
229	50	4	4	8	8	9	6	4	4	2	2		9	-	3	5	5	7	7		7
230	"	-	1	4	4	2	2	2	2	2	2		4	2	2	2	2	2	2		2
231	"	1	2	3	7	7	3	2	1	1	1		7	-	4	5	6	6	6		6
232	"	-	1	6	11	15	11	8	6	3	2		15	-	4	7	9	12	13		13
Total	200	5	8	21	30	33	22	16	13	8	7		35	2	13	19	22	27	28		28
233	25	1	1	2	3	3	4	3	3	1			5	1	1	2	2	4			4
234	"	2	2	4	6	5	4	3	0	0			6	1	2	3	6	6			6
Total	50	3	3	6	9	8	8	6	3	1			11	2	3	5	8	10			10
235	50	2	3	3	4	4	4	3	2	2	2		5	1	1	2	3	3	3		3
236	"	5	5	6	6	8	6	5	5	5	5		10	2	4	5	5	5	5		5

Tube No.	Initial Larval Inoc.	Numbers of Pupae: Number of days following inoculation											Total Pupae	Numbers of Adults: No. of days following inoculation							Total Adults
		11	12	13	14	15	16	17	18	19	20	21		15	16	17	18	19	20	21	
237	50	-	3	4	7	8	11	9	6	6	5		12	-	1	3	6	6	7		7
Total	150	7	11	13	17	20	21	17	13	13	12		27	3	6	10	14	14	15		15
238	50	-	1	1	1	1	2	2	2	2	2	2	3	-	1	1	1	1	1	1	1
239	"	-	1	3	4	6	10	8	8	8	5	4	12	-	1	3	3	4	7	8	8
Total	100	-	2	4	5	7	12	10	10	10	7	6	15	-	2	4	4	5	8	9	9
240	30	-	3	7	12	16	16	11	9	4	2	2	17	-	1	6	8	13	15	15	15
241	"	-	-	1	3	4	6	5	2	1	1	1	7	-	-	2	5	6	6	6	6
242	"	-	2	8	10	18	17	13	11	6	2	1	20	-	3	7	9	14	18	19	19
243	"	-	-	2	2	5	5	4	4	2	1	1	5	-	-	1	1	3	4	4	4
244	"	-	-	1	3	4	6	5	4	3	1	0	6	-	-	1	2	3	5	6	6
245	"	-	1	4	7	12	17	15	13	11	4	2	18	-	1	3	5	7	14	16	16
246	"	-	1	2	6	9	13	12	8	4	2	1	14	-	-	2	6	10	12	13	13
247	"	-	2	5	5	7	9	6	5	2	1	0	10	-	1	4	5	8	9	10	10
Total	240	-	9	30	48	75	89	71	56	33	14	8	97	-	6	26	41	64	83	89	89
248	30	-	2	5	9	14	16	11	7	5	1	0	18	-	2	7	11	13	17	18	18
249	"	-	1	4	7	12	15	14	9	7	4	2	17	-	1	3	8	10	13	15	15
250	"	-	1	1	4	7	8	7	6	3	1	1	9	-	1	2	3	6	8	8	8

Tube No.	Initial larval inoc.	Numbers of Pupae: Number of days following inoculation 12	13	14	15	16	17	18	19	20	21	Total pupae	Numbers of Adults: No. of days following inoculation 16	17	18	19	20	21	Total Adults
251	30	1	3	5	8	12	8	6	5	2	0	12	-	4	6	7	10	12	12
252*	"	-	-	1	1	2	2	1	1	1	1	2	-	-	1	1	1	1	1
253	"	3	4	6	7	9	9	7	6	6	4	14	2	5	7	8	8	10	10
254	"	1	3	5	7	9	7	6	4	1	0	10	1	3	4	6	9	10	10
Total	180	9	20	36	55	69	56	41	30	15	7	80	7	24	39	50	65	73	73
255	30	-	-	-	-	-	-					0	-	-					0
256	"	-	-	-	-	-	-					0	-	-					0
257	"	-	-	-	-	-	-					0	-	-					0
258	"	-	-	-	-	-	-					0	-	-					0
259	"	-	-	-	-	-	-					0	-	-					0
260	"	-	-	-	-	-	-					0	-	-					0
261	"	-	1	1	1	1	0					1	-	1					1
262	"	-	-	-	-	-	-					0	-	-					0
Total	240	-	1	1	1	1	0					1	-	1					1
263	25	-	-	-	1	1	1	1				1	-	-	-				0
264	"	-	1	2	3	3	3	4				4	-	-	-				0
265	"	-	-	-	-	-	-	-				0	-	-	-				0

Tube No.	Initial Larval Inoc.	Numbers of Pupae — Number of days following inoculation											Total Pupae	Numbers of Adults — No. of days following inoculation								Total Adults
		13	14	15	16	17	18	19	20	21	22	23		16	17	18	19	20	21	22	23	
266	25	-	1	1	1	1	1						1	-	-	-						0
Total	100	1	3	5	5	5	6						6	-	-	-						0
267	25	1	1	1	0	0	0						1	1	1	1						1
268	"	-	3	5	6	7	6						7	-	-	1						1
269	"	-	1	3	5	5	4						5	-	-	1						1
270	"	1	3	4	6	5	3						6	-	1	3						3
271	"	-	2	3	5	5	5						5	-	-	-						0
272	"	-	1	3	6	6	5						6	-	-	1						1
Total	150	2	11	19	28	28	23						30	1	2	7						7
273	30	-	1	3	4	7	16	12	9	7	3	3	17	-	-	1	5	8	10	14	14	14
274	"	-	3	6	12	14	15	9	7	4	2	2	17	-	-	2	8	10	13	15	15	15
275	"	-	-	1	4	9	10	9	7	3	1	0	10	-	-	-	1	3	7	9	10	10
276	"	-	1	3	3	7	8	8	7	6	6	6	11	-	-	2	3	4	5	5	5	5
277	"	-	2	2	5	12	11	10	5	3	2	2	12	-	-	1	2	7	9	10	10	10
278	"	-	-	2	5	6	7	7	4	2	1	0	8	-	-	1	1	4	6	7	8	8
279	"	-	3	5	8	12	11	6	6	4	3	1	13	-	-	2	7	7	9	10	12	12
280	"	-	1	3	5	8	7	6	4	3	1	1	8	-	-	1	2	4	5	7	7	7

Tube No.	Initial Larval Inocs.	Numbers of Pupae: Number of days following inoculation										Total Pupae	Numbers of Adults: No. of days following inoculation						Total Adults
		14	15	16	17	18	19	20	21	22	23		18	19	20	21	22	23	
Total	240	11	25	46	75	85	67	49	32	19	15	96	10	29	47	64	77	81	81

		Number of days following inoculation													No. of days following inoculation									
		18	19	20	21	22	23	24	25	26	27	28	29		22	23	24	25	26	27	28	29	
281	50	1	1	2	2	2	3	2	2	1				4	1	1	2	2	3				3
282	"	-	-	1	1	1	1	0	0	0				1	-	-	1	1	1				1
283	"	-	-	1	1	1	1	1	1	1				1	-	-	-	-	-				0
284	"	1	1	1	2	2	2	2	1	1				3	1	1	1	2	2				2
285	"	1	1	2	2	4	4	4	3	2				5	1	1	1	2	3				3
Total	250	3	3	7	8	10	11	9	7	5				14	3	3	5	7	9				9
286	50	-	-	-	-	-	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-	0
287	"	-	-	-	-	-	1	3	9	7	7	7	7	9	-	-	-	-	2	2	2	2	2
288	"	-	-	2	6	9	9	10	9	9	7	7	7	10	-	-	-	1	1	3	3	3	3
289	"	-	-	-	-	1	2	7	7	6	6	5	3	7	-	-	-	-	1	1	2	4	4
Total	200	-	-	2	6	10	13	21	26	23	21	20	18	27	-	-	-	1	4	6	7	9	9